OPTIMIZATION OF REGENERATION AND *AGROBACTERIUM* MEDIATED TRANSFORMATION OF WHEAT (*Triticum aestivum* L. cv. Yüreğir 89)

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

OPTIMIZATION OF REGENERATION AND *AGROBACTERIUM* MEDIATED TRANSFORMATION OF WHEAT (*Triticum aestivum* L. cv. Yüreğir 89)

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The objective of this study was to optimize regeneration parameters of immature inflorescence culture of *Triticum aestivum* cv. Yüreğir-89. The effects of dark incubation period and explant region on regeneration success were tested. Immature inflorescences were cut into 3 pieces as tip, mid, base and put onto 2mg /L 2,4-dichlorophenoxyacetic acid containing callus induction medium. These explants were taken to regeneration after 6, 9, 13 weeks of dark incubation period. The regeneration capacities of calli were determined as rooting and shooting percentages. Shooting percentages were found to be 72.0 % for 6 weeks of dark incubation and 64.1 % for 9 weeks of dark incubation while it decreases to 26.1 % in 13 weeks of dark incubation period. This showed that prolonged dark incubation period decreased regeneration capacity of the callus. There was no significant difference in regeneration capacities of tip, mid and base regions of immature

inflorescences, which reveals the potential of every region of inflorescence to be used as explant source in further transformation studies.

Besides regeneration studies, optimization of transformation parameters for Turkish wheat cultivar Yüreğir by using *Agrobacterium tumefaciens* AGLI containing binary vector pALl56 was performed. Transformation efficiencies were determined by monitoring the transient expression of *uid*A gene via histochemical GUS assay. Three to four weeks old calli were found to be more responsive to *Agrobacterium*-mediated transformation. Different media were tested for utilization during co-cultivation period. It was found that including phenolic compound acetosyringone along with ascorbic acid as an antioxidant was essential for succesful transformation.

Keywords: Wheat immature inflorescence; Regeneration; *Agrobacterium tumefaciens*; GUS; Transient gene expression

ÖZ

BUĞDAYDA (*Triticum aestivum* L. cv. Yüreğir 89) REJENERASYONUN VE AGROBAKTERİUMA DAYALI TRANSFORMASYONUN OPTİMİZASYONU

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Bu çalışmada Yüreğir ekmeklik buğday çeşidinde (*Triticum aestivum*) olgunlaşmamış başak taslağına dayalı kültürlerin rejenerasyon parametrelerinin optimize edilmesi amaçlanmıştır. Karanlıkta inkübasyon süresinin ve eksplant bölgesinin rejenerasyon başarısına etkisi test edilmiştir. Olgunlaşmamış başak taslakları, uç, orta ve dip olmak üzere üçe kesilmiş ve 2mg /L diklorofenoksiasetik asit içeren kallus oluşum besiyerine konulmuştur. Eksplantlar 6, 9, ve 13 hafta karanlık inkübasyon sürelerinden sonra rejenerasyon ortamına alınmıştır. Kallus rejenerasyon kapasitesi, köklenme ve çimlenme yüzdeleri olarak belirlenmiştir. Çimlenme yüzdeleri 6 hafta karanlık inkübasyonunda % 72.0 ve 9 hafta karanlık inkübasyonunda %64.1 olarak bulunmuş; buna karşın 13 hafta karanlık inkübasyon süresinde % 26.1'e düşmüştür. Bu, uzun süren karanlık inkübasyonunun kallus rejenerasyon kapasitesini azalttığını göstermektedir. Başak taslağının uç, orta ve dip bölgelerinin rejenerasyon kapasiteleri arasında anlamlı

bir fark gözlenmemiştir, ki bu da başak taslağının her bölgesinin daha sonra yapılacak gen aktarımı çalışmalarında eksplant kaynağı olarak kullanılabileceğini göstermektedir.

Rejenerasyon çalışmalarının yanısıra, Yüreğir ekmeklik buğday çeşidine pAL156 ikili vektörünü içeren *Agrobacterium tumefaciens* AGLI suşu ile gen aktarımı parametreleri optimize edilmiştir. Transformasyon etkinliği *uid*A geninin geçici ifadesi takip edilerek histokimyasal GUS tayini ile belirlenmiştir. Üç-dört haftalık kallusların Agrobakteriuma dayalı transformasyona iyi cevap verdiği bulunmuştur. Bakteri ve bitkinin birlikte inkübasyonu esnasında çeşitli besiyerleri test edilmiştir. Fenolik bileşik asetosiringonun ve antioksidant olarak askorbik asitin birlikte inkübasyon esnasında kullanılmasının başarılı transformasyon için gerekli olduğu saptanmıştır.

Anahtar kelimeler: Buğday, Başak taslağı, Rejenerasyon, *Agrobacterium tumefaciens,* GUS, Geçici gen ifadesi.

To millions of people suffering from hunger

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2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	Analysis of variance
bp	Base pair
cv	Cultivated variety
EDTA	Ethylenediamine tetra acetic acid
GUS	β-glucuronidase
HCl	Hydrogen chloride
HMW-GS	High molecular weight glutenin subunits
LEA	Late Embryogenesis Abundant
MS	Murashige-Skoog basal salt medium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OD	Optical density
PEG	Polyethylene glycol
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
spp.	Species
YEB	Yeast Extract Broth

CHAPTER I

INTRODUCTION

The cultivation of wheat (*Triticum* spp.) reaches far back into history. Wheat was one of the first domesticated food crops and for 8 000 years has been the basic staple food of the major civilizations of Europe, West Asia and North Africa. Today, wheat is grown on more land area than any other commercial crop and continues to be the most important food grain source for humans. Its production leads all crops, including rice, maize and potatoes (Curtis, 2002).

1.1. Characteristics of Wheat

1.1.1. Wheat

Wheat is an annual grass belonging to the family Gramineae (Poacea) and genus *Triticum*. Wheat has perfect flowers and reproduces sexually as a self-fertilizing (self-pollinating) crop. Some cross-pollination occurs, but usually this is less than 3 percent (Cook *et al.*, 1993). The plant is made up of root and shoot systems. The shoot is made up of a series of repeating units or phytomers, each potentially having a node, a leaf, an elongated internode and a bud in the axil of the leaf. The shoot is terminated by a spike, which is also called as head or ear, bearing about 20 spikelets. In the spike, the phytomer is made up of the spikelet (the axillary bud) and the rachis (node and internode). Each leaf comprises a cylindrical sheath which wraps the subtending leaf, and a lamina (blade). The wheat plant has the ability to tiller, *i.e.* to produce lateral branches. At the end of the vegetative phase

of development, the plant will consist of, in addition to the main shoot, a number of tillers (Kirby, 2002).

Wheat may exhibit either a winter or spring growth habit. Winter wheats are planted in the autumn and produce grain the following spring or summer. They require a vernalization period of temperatures near or slightly below freezing as well as minimum accumulation of growing degree days and or length of daylight to concert from vegetative to reproductive growth. Accumulated growing degree days are the total number of days average temperature above 0°C. Spring wheats are planted in the spring and produce grain the following summer. They require a minimum number of accumulated growing degree days and or length of daylight but not a vernalization period to convert from vegetative to reproductive growth (Cook *et al.*, 1993).

The seed, grain or kernel of wheat (botanically, the caryopsis) is the fruit of the plant and is normally about 4-8 mm long, depending on the variety and condition of growth. The embryo or germ is situated at the point of attachment of the spikelet axis, and the distal end has a brush of fine hairs. The embryo is made up of the scutellum, the plumule (shoot) and the radicle (primary root). The plumule, which forms the shoot when the seed germinates, has a stem attached to it and to the coleoptile, which functions as a protective sheath. The scutellum is the region that secretes some of the enzymes involved in germination and absorbs the soluble sugars from the breakdown of starch in the endosperm. Surrounding the endosperm is a metabolically active layer of cells or the aleurone layer, the seed coat (testa) and the fruit coat (pericarp). The outer material down to and including the aluerone layer is called bran (Cornell and Hoveling, 1998). The structure of wheat kernel is demonstrated in Figure 1.1.

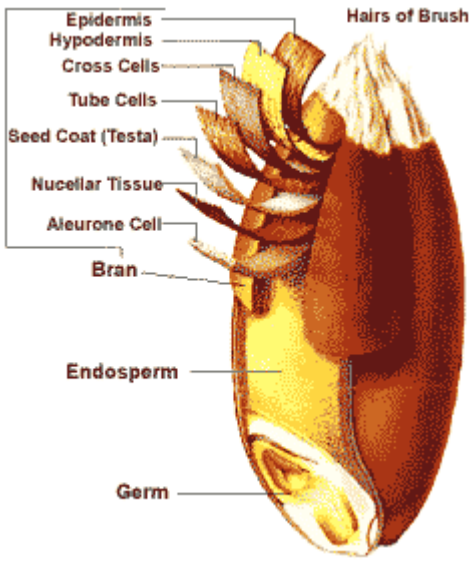


Figure 1.1. Structure of a wheat kernel
(Adopted from http://www.thebreadery.com/nutrition_info/nutrition_info.htm, Last access date: 28.09.2004).

1.1.2. Origin, Evolution and Classification of wheat

Wheat is a member of the grass family within the monocotyledonae sub-class and Angiospermae class. Within the grass family, wheat is a member of the tribe Triticeae and the genus *Triticum* (Cook *et al.*, 1993).

The wheats of current commerce (durum, common and club wheats) are products of natural hybridization of ancestral types. The best known ancestor is einkorn wheat (*Triticum monococcum* L.) (Schmidt, 1974). Millions of years ago, the first hybridization event is thought to have occurred when the wild grass *Aegilops speltoides* crossed with the wild diploid wheat, *Triticum monococcum* (Tan, 1985). The resultant hybrid was the tetraploid emmer wheat, *Triticum dicoccum*.

Domestication of emmer wheat lead to the evolution of the durum wheat. Hybridization of tetraploid durum wheat, *Triticum turgidum* var. *durum* (2n=28, AABB) with the diploid wild goat grass, *Aegilops tauschii,* led to the origin and evolution of hexaploid wheat about 8000 years ago (Patnaik and Khurana, 2001). The wild, primitive cultivated and modern cultivated wheats are shown given in Table 1.1.

Table 1.1. Names of wild, primitive cultivated and modern cultivated wheats

(Feldman, 1976).

Wild wheats	Primitive cultivated wheats	Modern cultivated wheats
T. monococcum var. *boeoticum* diploid (AA) *T. tauschii* diploid (DD) *T. turgidum* var. *dicoccoides* tetraploid (AABB) *T .timopheevii* tetraploid (AADD) *T. aestivum* hexaploid (AABBDD)	*T. monococcum* var. *monococcum*, einkorn (hulled) diploid (AA) *T. turgidum* var. *dicoccum*, emmer (hulled) *T. turgidum* var. *durum* (hull-less) tetraploid (AABB) *T. aestivum* var. *spelta* (hulled) hexaploid (AABBDD) *T. aestivum* var. *compactum* (hull-less) hexaploid (AABBDD) *T. aestivum* var. *aestivum* (hull-less) hexaploid (AABBDD)	*T. turgidum* var. *durum*, durum (hull-less) tetraploid (AABB) *T. aestivum* var. *spelta*, spelt (hulled) hexaploid (AABBDD) *T. aestivum* var. *compactum*, club wheat (hull-less) hexaploid (AABBDD) *T. aestivum* var. *aestivum*, common wheat (hull-less) hexaploid (AABBDD)

The geographic centre of origin of wheat is considered to be western Iran, eastern Iraq, and adjacent southern and eastern Turkey (Cook *et al.,* 1993). Some of the first domesticated primitive wheats as diploid *Triticum monococcum* (einkorn), a tetraploid *Triticum turgidum* var. *dicoccum* (emmer), and a hexaploid known as spelt (*T. aestivum* var. *spelta*) grown in the Fertile Crescent, the area in the Middle East, which stretches from Israel and Lebanon into Syria, Turkey, Iraq and Iran (Patnaik and Khurana, 2001). Presence of pure strands of wild diploid einkorn and wild tetraploid emmer wheats in these regions suggest that they may have been harvested and cultivated as such (Gill and Friebe, 2002). Recent genetic evidence indicates that einkorn wheat (*T. monococcum*) may have been domesticated from wild einkorn wheat (*T. monococcum* ssp. *aegilopoides)* in the region of the Karacadağ Mountains in southeast Turkey (Heun *et al*., 1997).

In Turkey, 10 different Triticum species, namely *T. baeoticum*, *T. monococcum* L., *T. timopheevi*, *T. dicoccoides*, *T. dicoccon*, *T. durum*, *T. turgidum*, *T. polonicum*, *T. carthlicum*, *T. aestivum* are grown naturally. *T. durum* Desf. (2n=48) and *T. aestivum* L. (2n=42) are the two most commonly cultivated wheats in Turkey today (Tan, 1985).

Today all commercially grown wheat species are hexaploid or tetraploid. The grain of many primitive cultivated wheat species, including einkorn, emmer and spelt, is hulled, whereas the grain of all modern wheat species, including durum, club and common wheat is free-treshing (Cook *et al.,* 1993). The free-treshing, compact eared and spherical grained wheat forms are thought to arise from primitive hexaploid forms as a result of mutations (Miller, 1987).

1.1.3. Genetic and cytogenetic characteristics of wheat

The basic number of chromosomes in wheat species is seven. Thus, diploid wheat species have 14 chromosomes, the tetraploid emmer and modern durum wheat species have 28 chromosomes and the common hexaploid wheat species have 42 chromosomes (Cook *et al.*, 1993).

The diploid wheats comprise a genomic group with the genome formula AA. This basic genome is also common to all the polyploid wheats (Miller, 1987). There are other different wild diploid species whose genomes have been labeled by cytologists as BB, CC, DD etc. Through natural hybridization, one diploid species combine its set of chromosomes with a different set of chromosomes of another diploid species by a process known as amphidiploidy. Tetraploid wheat species arose as a consequence of such natural crosses between two different diploid wheat species (Cook *et al.*, 1993). Hexaploid wheat species arose by the combination of a tetraploid genome AABB with diploid genome DD of *Ae. squarrosa* to produce a hexaploid hybrid of genome AABBDD (Miller, 1987).

Triticum aestivum, the bread wheat, has a genome size of 16 billion base pairs (bp) of DNA organized into 21 pairs of chromosomes, seven pairs belonging to each of the genomes A, B and D, which is designated as 2n = 6x = 42, AABBDD (Gill and Friebe, 2002).

1.1.4. Nutritional value of wheat

In many countries, wheat is the major component of the diet. It is non-perishable, easy to store and transport, has a good nutritional profile and allows the manufacture of a wide variety of satisfying products. For many people, wheat-based foods are the major source of energy, protein and various vitamins and

minerals. In some population groups, wheat-based foods provide two-thirds or more of the daily caloric intake (Ranhotra, 1994).

The composition of the kernel varies widely in wheat. For example, usual range of protein is from 8% to 15 %, however 7% or as high as 24 % can occur. The embryo contains 26 % protein whereas the aleurone contains 24 % protein. From the endosperm a water insoluble protein fraction called gluten can be isolated. It is particularly important for the leavening in bread making (Inglett, 1974).

When protein contribution of wheat is considered, as in all other grains, lysine is the most deficient amino acid in wheat. The amino acid profile of wheat improves dramatically when only a small quantity of leguminous or animal protein is simultaneously included in the diet. Wheat itself is low in fat and the fat that is present is high in unsaturated fatty acids which lower elevated blood cholesterol levels (Ranhotra, 1994). Triglycerides, phospholipids and glycolipids, fatty acids, sterols, monoglycerides and diglycerides are present in whole wheat as well as endosperm lipids. Tocopherols are another class of lipid substances present in wheat. Wheat germ is an abundant source of α-tocopherol, which is known as vitamin E (Inglett, 1974).

Whole-wheat flour and its bran are a good source of fiber, particularly water insoluble fiber. In contrast, white flour, although not high in total fiber, is relatively high in soluble fiber. Due to high fiber content and associated feeling of fullness, wheat based products can help dieter control of food intake and weight loss when accompanied with restricted caloric intake at the same time (Ranhotra, 1994). Wheat has a relatively high content of thiamine and niacin compared to other cereal grains. It is low in riboflavin. Niacin and riboflavin are not affected by heat so much and thus are not destroyed in bread making process (Inglett, 1974).

The only problems associated with wheat plant and its products is the allergenicity of some people to gluten as a food, and the allergenicity of some people to wheat

pollen in the air. The extent of these problems is well understood (Cook *et al.*, 1993).

Societies consuming diets high in grain-based foods show a lower incidence of chronic degenerative diseases such as heart disease, colon cancer and diabetes. Grain based foods are economical foods as compared to most other foods. It is suggested that in societies where nutrient deficiencies are still a major health problem, grain-based foods provide an effective vehicle to fortify foods (Ranhotra, 1994).

1.1.5. Types of wheat and their uses

Wheat is classified with respect to different basis according to its agronomic and end-use attributes. These classifications are generally based on quality, color and growth habit (Oleson, 1994). Although useful as a livestock feed, wheat is used mainly as a human food due to its nutritious, concentrated, easily stored and transported nature, and ability to be easily processed into various types of food. Unlike any other plant-derived food, wheat contains gluten protein, which enables leavened dough to rise by forming minute gas cells that hold carbon dioxide during fermentation (Gibson and Benson, 2002).

Approximately, 90 to 95 percent of the wheat produced in the world, is common wheat (*T. aestivum*), which is classified as hard wheat or soft wheat, depending on grain hardness. Wheat is utilized mainly as flour (whole grain or refined) for the production of a large variety of leavened and flat breads, and for the manufacture of a wide variety of other baking products. The rest is mostly durum wheat (*T. durum*), which is used to produce semolina (coarse flour), the main raw material of pasta making (Peña, 2002). The types and utilizations of wheats is given in Table 1.2.

Table 1. 2. Types and utilizations of wheat

(Adopted from http://wbc.agr.state.mt.us/prodfacts/usf/usclass.html#, 28.09.2004)

HARD RED WINTER WHEAT	Has wide range of protein content, good milling and baking characteristics. Used to produce bread, rolls and, to a lesser extent, sweet goods
HARD RED SPRING WHEAT	Contains the highest percentage of protein, making it excellent bread wheat with superior milling and baking characteristics.
SOFT RED WINTER WHEAT	Used for flat breads, cakes, pastries, and crackers.
HARD WHITE WHEAT	Closely related to red wheats, this wheat has a milder, sweeter flavor, equal fiber and similar milling and baking properties. Used mainly in yeast breads, hard rolls, bulgur, tortillas and oriental noodles.
SOFT WHITE WHEAT	Used in much the same way as Soft Red Winter (for bakery products other than bread). Contains low protein, but has high yielding. Used for production of flour for baking cakes, crackers, cookies, pastries, quick breads, muffins and snack foods.
DURUM WHEAT	Used to make semolina flour for pasta production. Common foods produced from durum wheat are macaroni, spaghetti, and similar products.

Besides being the major ingredient in most breads, rolls, crackers, cookies, biscuits, cakes, doughnuts, muffins, pancakes, waffles, noodles, pie crusts, ice cream cones, macaroni, spaghetti, puddings, pizza, and many prepared hot and

cold breakfast foods, wheat it is also used in baby foods, and is a common thickener in soups, gravies, and sauces. Much of the wheat in the form of by-product of the flour milling industry is used for livestock and poultry feed. Wheat straw is used for livestock bedding (Gibson and Benson, 2002). About 6 % of wheat is used for industrial purposes which include the processing of wheat into starch and gluten for widely range of utilization in processed food products, the production of ethyl alcohol, plastics, varnishes, soaps, rubber and cosmetics (Olenson, 1994). The straw may be used for newsprint, paperboard, and other products.

1.1.6. Wheat production

Wheat has the widest adaptation of all cereal crops and is grown in some 100 countries around the world (Oleson, 1994). According to the "Grain Market Report" figures of International Grains Council which is released in January, 2003, top wheat producing nations are China, India, Russia, United States and France. The amount of wheat produced by top 10 producers and total amount of wheat produced in the world from 1996 to 2002 is given in Table 1. 3.

According to United Nations Food and Agricultural Organization (FAO) 2002 reports, wheat production in the world is about 568 million tons and China, India, Russia, USA and France have produced 51.7 % of world's wheat. FAO figures indicate that Turkey has a 3.5 % of world wheat production and rank eightieth with a close efficiency to world's average. Again according to FAO, in 2002, wheat was cultivated in 9.400.000 hectares of area, and 20 million tons of wheat is produced with an efficiency of 21.277 kg/Ha. However, this amount is not sufficient for the national demand for wheat. In Union of Turkish Chambers of Agriculture 2004 Wheat Report, it was indicated that although utilization of wheat in developed countries is not so high, wheat based nutrition and high demand for wheat is common in Turkey and in countries where gross national income per

capita is low. It was also mentioned that in Turkey 60% of daily calorie requirement was acquired from wheat.

Thus, wheat is also imported from other countries. Bread wheat is one of the highly imported crops in Turkey and when the average of year 1996 to 2001 is considered, it has a 9.71 % share in mostly imported crops with a value of 245.1 million dollars (Dölekoğlu, 2003).

Table 1.3. Top wheat producing nations (1996-2002), in terms of million tons.

Country	2002	2001	2000	1999	1998	1997	1996
China	89.0	94.0	99.7	113.9	109.7	123.3	110.6
India	71.5	68.8	76.4	70.8	65.9	69.4	62.6
United States	44.0	53.3	60.8	62.7	69.4	67.5	62.0
France	39.0	31.4	37.5	37.2	39.8	33.9	35.9
Russia	50.6	46.9	34.5	31.0	27.0	44.3	34.9
Canada	15.7	20.6	26.8	26.9	24.1	24.3	29.8
Germany	20.8	22.8	21.6	19.6	20.2	19.8	18.9
Pakistan	19.5	19.1	21.1	17.9	18.7	16.7	16.9
Australia	18.5	24.1	18.5	24.1	22.1	19.4	23.7
Turkey	17.5	15.5	17.5	16.5	18.5	16.2	16.2
WORLD TOTAL	**563.2**	**579.2**	**582.3**	**584.7**	**586.9**	**610.0**	**582.4**

1.2. Improvement of wheat

The increase in the demand for food production as the world population enlarges cannot be disregarded. Hoisington *et al.* (2002) stated the requirement for food in next decades as: “Two hundred people are being added to the planet every minute. It is forecast that by the year 2050, the world’s population will double to nearly 12

billion people. To feed this population, these people will require a staggering increase in food production. In fact, it has been estimated that the world will need to produce more than twice as much food during the next 50 years as was produced since the beginning of agriculture 10 000 years ago". As being the mostly consumed crop in the world, wheat production should be improved either by increasing the yield in areas where wheat has already being produced or by enlarging the wheat cultivated area via releasing cultivars that can be grown in marginal environments.

In Turkey, wheat improvement studies concern the improvement of bread wheat and durum wheat. The conducted researches are mainly focused on resistance to diseases, adaptivity to the region and climate, yield and quality. Wheat is susceptible to many pests and diseases, especially rust, bunt and sunn pest are the main problems encountered in Turkey. There are some ongoing studies for developing sunn pest resistant transgenic wheat plants with Turkish varieties (Ebru Özgür, personal communication). Also, developing plants against zinc deficiency which is seen especially in Central Anatolia (Çakmak *et al.*, 1999) would be very suitable. As far as we know, no researches have been conducted for improving nutritional characteristics of wheat. However, since there is a high demand for wheat and wheat covers very high percentage of daily calorie requirement in diet of Turkish people, increasing its nutritional value is a very promising way of fortifying foods.

1.2.1. Wheat cultivation

Scientific approaches to crop improvement have their history in the rediscovery of Mendel's law at the beginning of this century. Following Mendel's law, breeders have been searching for technologies and converting this 'art' of breeding to a 'science'. Wheat breeders have been able to introduce desirable traits that increased the grain yield and minimize the crop loss (Patnaik and Khurana, 2001).

The conventional breeding techniques utilize processes of crossing, back crossing and selection. The main breeding schemes of wheat comprise the development of three types of wheat cultivars: pure lines, multilines, and hybrids. Pure lines are produced by cross-breeding followed by selection until the line is genetically uniform and its duration is usually eight to ten generations. Multilines are mixtures of pure lines. Hybrids are produced by either the cytoplasmic male-sterile method or by the chemical hybridization agent method. Pure-line crossbred cultivars are the most common ones (Cook *et al.*, 1993).

The tribe Triticeae comprising of over 300 species including wheat, rye and barley is considered as germplasm source for wheat improvement by classical breeding approaches (Patnaik and Khurana, 2001). However, much of the world's wheat germplasm possesses traits that are unsuitable for modern processing of wheat grain for various food products. Use of exotic germplasms for cultivar improvement can be done but it requires many generations of backcrosses and selection. Another option is to use of relatively adapted parental stocks with acceptable quality traits, however this preference from narrow crosses reduces opportunities for significant advances in yield and other complexly inherited traits (Cook *et al.*, 1993).

Many of the necessary or desirable characteristics of wheat needed for solving specific production problems or meeting specific market of nutritional needs cannot be achieved by traditional breeding technologies due to the lack of availability of adequate resistance to many important diseases and insect pests in the existing wheat germplasm bank. Accordingly, the efforts of plant breeders in the improvement of wheat yield by using conventional breeding methods attained a plateau especially in terms of yield (Sahrawat *et al.*, 2003).

Moreover, conventional breeding techniques which are based on processes of crossing, back crossing and selection, are time consuming and could hardly keep pace with the rapid co-evolution of pathogenic micro-organisms and pests. Thus,

the development of *in vitro* technologies have complemented the conventional methods of wheat breeding for the production of novel cultivars with desirable characters (Patnaik and Khurana, 2001).

1.2.2. Wheat biotechnology

Biotechnology, which involves the systematic application of biological processes for the beneficial use, is emerging as one of the latest tools of agricultural research in recent years. Besides traditional plant breeding practices, biotechnology contributes to the development of new methods to genetically alter and control plant development, plant performance and plant products. Since plant biotechnology involves the delivery, integration and expression of defined genes into plant cells which can regenerate into whole plants, biotechnological approaches have the potential to complement conventional methods of breeding by reducing the time taken to produce cultivars with improved characteristics. Unlike conventional breeding which utilizes domestic crop cultivars and related genera as a source of genes for improvement of existing cultivars, biotechnological approaches can transfer defined genes from any organism and in this manner can increase the gene pool available for improvement (Patnaik and Khurana, 2001).

In the early 60's, conventional breeding techniques integrated with improved farm management practices led to a significant increase in world wheat production. However, later on, the outbreak of these practices has reached to a plateau which directed the targets of genetic improvement to minimize yield losses caused by various biotic and abiotic stresses. Biotechnology offers solutions for not only lowering production costs by making plants resistant to various abiotic and biotic stresses, but also by enhancing the product quality such as by improving the nutritional content or processing or storage characteristics of the end product.

The introduction of foreign genes encoding for useful agronomic traits into commercial cultivars has resulted in saving time required for introgression of the desired trait from the wild relatives by conventional practices and prevents the extensive use of hazardous biocides which destroys the environmental balances. In recent years, wheat improvement efforts have therefore focused on raising the yield potential, quality characteristics, resistance to biotic stresses and tolerance to abiotic stresses depending on the regional requirement of the crop (Patnaik and Khurana, 2001).

It is well known that the efficiency of a transformation protocol depends on several components. First component is the presence of a reliable regeneration method for the plant species into which the novel gene is desired to be introduced. Secondly, the presence of a suitable and highly efficient gene transfer technique is required. Finally, an effective screening and selection method for the recovery of transformants should be present. In order to attain these requirements, scientists from various parts of the world have focused not only on the delivery of foreign gene methods but also on the optimization of regeneration of wheat in tissue culture.

1.3. Tissue culture studies in wheat

Plant tissue culture methods provide development of suitable callus systems and offer efficient plant regeneration from cultured cells and tissues. These tissue culture applications can be used for the improvement of crop plants. In order to better explain the tissue culture practices employed in wheat, general information on callus cultures and plant regeneration systems is documented in this section.

Callus is a disorganized proliferated mass of actively dividing cells. A callus consists of a mass of loosely arranged thin-walled parenchyma cells arising from the proliferating cells of the parent tissue. Callus has the potential to develop

normal roots, shoots, and embryoids that can form plants. Plant materials used to initiate the callus, the composition of the medium, and the environmental conditions during the incubation period are the important factors affecting general growth characteristics of a callus (Dodds and Roberts, 1985).

The formation of callus from an explant roughly contains 3 stages: induction, cell division, and differentiation. During induction phase, the metabolism is prepared for cell division. At the actively cell division phase, the cells of the explant are reverted to meristematic or dedifferentiated state. Third phase is the appearance of cellular differentiation and expression of certain metabolic pathways (Dodds and Roberts, 1985). Due to the heterogeneous nature of tissue explants, only certain cell types are capable of responding to the *in vitro* culture conditions. Those cells are defined as competent. The term competence refers to the capability of a cell or group of cells to respond to an inductive stimulus for a developmental process (Ritcie and Hodges, 1993).

In vitro plant regeneration can be performed by organogenesis or somatic embryogenesis. Organogenesis is a developmental pathway in which shoots or roots (*i.e.* organs) have been induced to differentiate from a cell or group of cells. *In vitro* plant regeneration by organogenesis usually involves induction and development of a shoot from the explant tissue, with or without an intervening callus stage, followed by transfer to a different medium to induce root formation and development. If the shoot or root is induced and develops directly from a pre-existing cell in the explant without undergoing an initial callus phase, then the process is called as direct organogenesis. On the other hand, indirect organogenesis involves an initial phase of callus proliferation and growth, followed by shoot or root induction and development from this proliferated callus tissue (Ritcie and Hodges, 1993).

Somatic embryogenesis is a developmental pathway in which embryos have been induced to form from a somatic cell or group of somatic cells. Somatic

embryogenesis can occur directly without an intervening callus phase but indirect somatic embryogenesis is generally more common (Ritcie and Hodges, 1993).

In somatic embryogenesis, each developing embryoid passes through the sequential stages of embryo formation: following repeated cell divisions, cell aggregates progressively develop and pass through globular, heart, and torpedo stages before ultimately forming plantlets. Abnormalities such as embryonal budding and embryogenic clump formation might occur, if relatively high levels of auxin are present in the medium after the embryogenic cells have been differentiated. For this reason, two different media may be required for the initiation of the embryonic cells and for the subsequent development of these cells into embryoids. For some plants such as wheat, embryo initiation and maturation take place in the same medium (Dodds and Roberts, 1985).

Germination of somatic embryos is usually induced on hormone-free culture medium or medium that contains low levels of an auxin or low levels of both an auxin and a cytokinin. Under these conditions, the somatic embryos which are developmentally capable of germinating, do so without maturing. Unlike mature zygotic embryos, which germinate vigorously into sizeable plants within several days, germinating somatic embryos generally lack vigor and may require many days to develop into healthy, rapidly growing plants (Carman, 1995).

In 1980s, the much of the potential impact of advances in biotechnology used to be demonstrated in model plant species, particularly in the genus *Nicotiana* (tobacco and related species). As indicated in Vasil's review in 1987, until that time, there were only a few reports of reproducible plant regeneration from tissue cultures of the Gramineae. In most cases, even the induction and maintenance of long-term callus cultures was found to be extremely difficult and regenerative capacity was often lost within the first few subcultures. The induction and long-term maintenance of embryogenic tissue cultures in a wide variety of gramineous species was achieved by the utilization of 2,4-dichlorophenoxyacetic acid (2,4-D)

as the auxin type of plant growth regulator and by choosing the explants which contains meristematic and undifferentiated cells (Vasil, 1987).

Rapid and large scale clonal propagation of especially the ornamental plants is a widely employed plant regeneration technique. However, this technique is proven to be difficult for cereals and grasses. The regeneration of all major cereals in tissue culture is possible by the utilization of 2,4-D and the usage of immature embryos or young inflorescence or young leaf based explants via somatic embryogenesis. Owing to their origin form single cells, plants obtained from somatic embryos are non-chimeric and show genetic uniformity and clonal fidelity (Vasil, 1987).

1.3.1. Factors affecting *in vitro* culture of wheat

There are several factors that affect the *in vitro* culture of wheat. The type of explant, the genotype of the cultivar used and components of the tissue culture media are the most important ones.

The type of explant and the developmental stage of the explants have been found to be critical factors in the establishment of totipotent cultures. Embryos, leaves and inflorescences possess a brief period of time in their developmental stages during which they have competence to form embryogenic cultures (Botti and Vasil, 1984; Lu and Vasil, 1981; Vasil and Vasil, 1981). Their competency is due to their still meristematic nature and being not yet fully committed to specialized functions and might be due to their hormonal status. Vasil (1987) has suggested the likelihood of endogenous pools of plant growth regulators to be related to the gradient of embryogenic competence seen in young leaves, inflorescences of grass species and indicated that explants obtained before or after this developmental stage form only non-morphogenic or non-embryogenic callus.

A relationship between plant genotype and *in vitro* response particularly in terms of regeneration has been reported in several studies with cereals (Sears and Deckard, 1982; Maddock *et al.,* 1983; Mathias and Simpson, 1986; Borrelli *et al.*, 1991; Fennel *et al.*, 1996; Özgen *et al.*, 1998). Sears and Deckard, in 1982, investigated the genotypic differences in immature embryo based callus cultures. They have found that for most of genotypes, the degree of cellular organization and shoot meristems can be controlled by manipulation of 2,4-D concentration which indicates variation in tolerance to 2,4-D amongst genotypes. Also, Maddock *et al.* (1983) evaluated plant regeneration from immature embryo and inflorescence tissues of 25 wheat cultivars and they reported clear differences in morphogenic capacities of genotypes.

Vasil (1987) has indicated that the relationship of genotype to morphogenetic competence *in vitro* is complex and indirect. "This relationship is influenced by physiological and environmental factors and has a strong effect on the synthesis, transport and the availability of plant growth regulators" (Vasil, 1987). It is also suggested by Vasil that when suitable explants at defined developmental stages are excised from plants and cultured under optimal conditions with appropriate amount of plant growth regulators, even recalcitrant plants or genotypes can be induced for morphogenesis.

It can be concluded that besides type of explant and genotype of the cultivar, culture conditions and the amount of exogenously applied plant growth regulators which act simultaneously with the endogenous plant hormones have a deep impact on the fate of the explant in tissue culture.

1.3.2. Induction and maintenance of embryogenic callus

In wheat, embryogenic callus cultures can be obtained most readily from immature embryos, and young inflorescences and leaves. In embryos, callus originates from

epithelial and subepithelial cells of the embryo scutellum and in inflorescences mainly from the rachis and glumes of the young inflorescence (Ozias-Akins and Vasil, 1982).

During the initial period of excision and culture of explant in the presence of 2,4-D, embryogenic competence is expressed by a few cells. Somehow, these cells are selected and preferred. The maintenance of adequate levels of 2,4-D helps to perpetuate the embryogenic nature of cultures by continued divisions in embyogenic cells and in active meristematic zones formed in proliferating tissues. Lowering of 2,4-D levels results in the organization of somatic embryos. Embryogenic cells are characteristically small, thin-walled, tightly packed, richly cytoplasmic and basophilic, and contain many small vacuoles as well as prominent starch grains (Vasil and Vasil, 1981). When 2,4-D levels become too low, the embryogenic cells enlarge, develop large vacuoles, lose their basophilic and richly cytoplasmic character, walls become thicker, starch disappears (Vasil and Vasil, 1982). This irreversible process of differentiation leads to the formation of a friable non-embryogenic callus which is generally non-morphogenic or may form roots. Most cultures are actually mixtures of embryogenic and non-embryogenic cells as a result of such continuous conversion. In general, embryogenic calli are characterized as off-white, compact, nodular type and as white, compact type. Upon subculture the nodular embryogenic callus was defined to become aged callus and formed an off-white, soft and friable embryogenic callus both of which retain the embryogenic capacity for many subcultures (Redway *et al.*, 1990). In order to maintain the embryogenic potential, and to ensure that plant regeneration is solely via somatic embryogenesis, it is important to visually select and transfer embryogenic calli during subcultures.

1.3.3. Genetic variability in culture and in regenerated plants

Genetic variability during culture, which is common in plant tissue cultures, can be a great problem in the true clonal propagation of cereals and grasses where absolute fidelity to the genotype is required. Vasil (1987) proposes that the occurrence of permanent phenotypic changes is not a frequent phenomenon and some of the observed changes may be temporary or a result of a chimerism. There exists no large scale somaclonal variation in agronomically useful varieties of any cereal or grass species. As cited in Vasil (1987), one of the apparent observations reported by Vasil and co-workers in 1982 was phenotypic uniformity, normal chromosome number and absence of albinos in plants regenerated by somatic embryogenesis in Gramineae and no polyploids and aneuploids were found in any of the species. It is quite likely that there exists changes in small amounts involving the minor structural rearrangements of chromosomes, or transpositional events, point mutations and alterations in organelle DNA etc. However, detecting their occurrence is very difficult. In a report by Chowdhury and co-workers in 1994, no major variation detected in wheat mitochondrial DNA during short-term culture upon RFLP analysis. It might be also possible that the somatic embryos with important chromosomal aberrations would not develop until maturity to give rise to a plant and thus their occurrence have not been recorded. Some genetic variability may arise spontaneously in culture. The reason might be components of the nutrient medium, loss of controls of cell division and differentiation in intact tissues and plants and activity of transposable elements.

1.3.4. Studies on wheat regeneration systems

A variety of explants have been used in attempts to establish regenerable tissue cultures of wheat, including whole seed, mature and immature embryos, isolated scutellum, immature inflorescence, immature leaf, mesocotyl, apical meristem, coleoptilar node, and root (Rakszegi *et al.*, 2001). Among them the most widely

used explants are immature embryos, immature inflorescences, and mature embryos and a survey of the studies exploiting these explants is given below.

Immature embryos

One of the earliest reports on plant regeneration from immature embryo-derived callus cultures of wheat was reported by Ahloowia (1982). In this study, callus formation was found to be better in a medium which is devoid of kinetin but contains 2,4-D and IAA. The regenerated plants were stated to be originating via organogenesis but some bipolar structures with colorless root and green shoot zones were also reported.

Sears and Deckard (1982) compared callus induction and shoot formation from immature embryos of 39 cultivars of winter wheat and by using one defined series of media, they achieved high rates of immature embryo derived callus of some cultivars and found that both the rate and occurrence of regeneration was quite cultivar specific.

In 1983, Ozias-Akins and Vasil reported efficient somatic embryogenesis in tissue cultures derived from the scutellum of immature embryos of *T. aestivum*. They showed that callus maintained in the dark appeared equal to those in the light with respect to growth rate but superior with respect to formation of embryogenic tissue. They also showed microscopical and histological examination of shoot development.

In 1986, Mathias and Simpson who investigated the effect of the interaction of genotype and culture medium on the initiation of callus from immature embryos have found that regeneration from calli ranged from 0-60 % in eight lines and they concluded that the genotype clearly has more effect on the response of calli than the presence of complex organic additives in the media.

In 1991, Italian group Borrelli and co-workers have regenerated five varieties of Durum wheat as a result somatic embryogenesis of long term selective subculture of immature embryo derived embryogenic calli. They also reported the differences in genotypic response.

In 1993, Bai and Knott established long term culture from the immature embryos of a hybrid between *Triticum aestivum* L. and *Thinopyrum ponticum*. They found that with increasing time on a maintenance medium, the plant regeneration rates of the hybrid calli decreased when transferred to regeneration media containing 0.1, 0.2, or 0.5 mg/L 2,4-D. After 3 months of subculture, the highest plant regeneration rate was obtained on the medium containing 0.5 mg/L 2,4-D, while on the 24^{th} month of subculture the highest plant regeneration rate was obtained on the medium containing 0.1 mg/L 2,4-D. So, as the callus aged it was important to reduce the level of 2,4-D in the regeneration medium. They confirmed that after long term callus culture on a maintenance medium, the concentration of 2,4-D in the regeneration medium should be decreased to create optimum culture conditions.

Fennell and colleagues from International Maize and Wheat Improvement Center (1996) investigated the regeneration of elite bread wheat cultivars from immature embryos. They found the regeneration percentage to be varying widely between 2% and 94% with both genotype and the initiation medium utilized.

In 1996, Bommineni and Jauhar have described the establishment of optimal *in vitro* conditions for the rapid regeneration of durum wheat plantlets from isolated immature scutella. Upon trials of different solidifying agents, they routinely utilized agar solidified induction medium for rapid initiation of embryogenic calli and half strength hormone free medium as regeneration medium. The regeneration frequency was found to be variable among genotypes and the regeneration frequencies of cultivars ranged from 60-100 %, in their best responding conditions, with an overall mean of 71.2%.

In 1998, Machii and co-workers have screened Japanese wheat genotypes for high callus induction and regeneration capacity from anther and immature embryo cultures. For immature embryo culture, 97 genotypes out of 107 were found to show 90% callus induction rate and 74 of them regenerated to plants. The regeneration percentages of best responding genotypes were found as 90%, 80% and 75 %.

In 1999, Fernandez and co-workers have investigated the embryogenic response of immature embryo cultures of durum wheat and they showed that addition of 1 mg/ L of $AgNO_3$ enhanced the induction of somatic embryogenesis more than 22 fold and affect both the percentage of embryogenic explants and also the number of somatic embryos per explant.

Arzani and Mirodjagh (1999) have evaluated the response of twenty eight cultivars of durum wheat to immature embryo culture, callus induction and *in vitro* salt stress. They found significant differences among cultivars for potential of regeneration from immature embryo, and also concluded that fresh weight growth data is more effective than callus induction frequency in evaluation of callus induction. They also concluded that by using relative fresh weight growth in different NaCl concentrations, it was possible to select for more tolerant genotypes.

In 2001, Khanna and Daggard has reported a method which results in increased regeneration potential of ageing calli initiated from isolated scutella of immature embryos of nine elite Australian wheat cultivars by the application of spermidine and water stress. The calli of some cultivars up to 12-weeks old had shown improved regeneration with 16 h of dehydration stress. They also reported 3-50% increase in regeneration potential of older calli (16-20 weeks old) upon exogenous spermidine application while regeneration of younger calli (4-week old) was affected negatively by spermidine.

Pellegrineschi and co-workers (2004) have obtained optimal callus induction and plant regeneration in bread and durum wheat by manipulating NaCl concentration in the induction medium. For bread wheat, medium with 2.5 mg/L 2,4-D and for durum wheat, medium with 2 mg / L 2,4-D and 2 mg/L NaCl was found to yield higher regeneration responses. As also seen in the report of Khanna and Daggard (2001), water stress was found to affect somatic embryogenesis positively.

Mature embryos

Heyser *et al.* (1985) reported long-term and high frequency plant regeneration from *Triticum aestivum* by using mature and immature embryos. They proposed that it is possible to change the amounts of embryogenic and non-embryogenic callus in both mature and immature embryo derived cultures by altering the 2,4-D concentrations in the medium.

In 1996, Özgen and co-workers compared the callus induction and plant regeneration from immature and mature embryos of winter durum wheat genotypes. For mature embryos, they used endosperm supported culture. They found mature embryos to have a low frequency of callus induction but a high regeneration capacity. When availability, rapidity and reliability of mature embryo were considered, mature embryo culture was suggested by authors as an alternative to immature embryo cultures.

In a subsequent study, in 1998, Özgen and co-workers cultured immature and mature embryos of 12 common winter wheat genotypes and compared their regeneration response. They used endosperm-supported callus induction method to overcome the low frequency of callus induction associated with mature embryos. They concluded that callus responses of mature embryo culture may be higher than those of immature embryo culture. They found no significant correlations between callus induction, regeneration frequency and number of plants regenerated. They

also confirmed strong genotypic influences on callus induction and regeneration capacity.

Delporte and co-workers, in 2001, regenerated plants from thin mature embryo fragments of wheat. They used small fragments of mature embryo (with 500µm mean diameter) as explants to initiate embryogenic calli on solid medium supplemented with 10µM 2,4-D and ended up with 90 % callus induction frequency. They obtained highest embryogenic callus induction rate when 2,4-D is suppressed after a 3-4 week induction period. The optimal protocol was found to produce 25-30 plants per 100 embryos.

In 2003, Zale and co-workers have compared the behaviour of a diverse number of wheat genotypes in their tissue culture response by using mature embryos as the explant source. Besides 47 wheat cultivars, they also tested the tissue culture response of common wheat progenitors *Triticum monococcum*, *Triticum tauschii* and *Aegilops speltoides*. Among the progenitors, the authors indicated the higher regeneration capacity of *Ae. speltoides*. They found 39 % regeneration capacity as the mean of cultivars and 31% culture efficiency in mature embryos of 29 hexaploid wheat cultivars and lines. They also compared immature embryos and mature embryos of elite wheat cultivars and found immature embryos to be superior in terms of regeneration. On the other hand, they concluded that mature embryos can produce sufficient amount of regenerants by saving for growth facility resources and time.

Özgen's group (2001) has also reported the positive effect of cytoplasm in callus induction, regeneration capacity of callus, culture efficiency and numbers of regenerated plants.

Immature inflorescences

In 1982, Ozias-Akins and Vasil have reported that tissue cultures of *Triticum aestivum* initiated from young inflorescences and immature embryos possessed the potential for regeneration to whole plants. They stated that both friable and compact type of callus were produced on MS with 2 mg / L 2,4-D. Embryogenic callus from inflorescence tissue of wheat was found to have the same morphology as that arising from the scutellum of immature embryos and could organize somatic embryos which germinate to form plants. They suggest that while typical bipolar embryos are generally not formed, plant regeneration nevertheless takes place through embryogenesis and the precocious germination of the embryoids.

In their study of regeneration of 25 cultivars of wheat from cultured immature embryos and inflorescences, Maddock and co-workers have shown in 1983 that the capacity for plant regeneration differs consistently between cultivars of wheat and is under genetic control. They tested the regeneration response of 19 wheat genotypes and found that the percentage of shoot forming cultures ranged from 29 to 100 % for immature inflorescences while it ranged from 12 % to 96% for immature embryos.

In 1990, Redway and colleagues from Vasil's laboratory have identified the callus types in commercial wheat cultivars. They found immature embryos to be more suitable for embryogenic callus formation while anthers responded poorly and inflorescences gave an intermediate response. They also reported that there were no significant differences in regeneration response of calli derived from embryos and inflorescences cultured on different initiation media.

In 1999, Barro and co-workers optimized media for efficient somatic embryogenesis from immature inflorescences and immature scutella of elite wheat, barley and tritordeum cultivars. They suggest that for wheat and tritordeum inflorescences, regeneration from embryogenic calli induced on medium with

picloram was almost twice as efficient as regeneration from cultures induced on 2,4-D. They also demonstrated that embryogenic capacity from inflorescences with an average of 92 % was higher than from immature scutella for which average is 62 %; however, shoot regeneration from scutella was clearly higher than inflorescences.

Caswell and colleagues (2000) regenerated fertile plants from immature inflorescence explants from each of four Canadian wheat cultivars. They have tested two different media: MS based medium which contains 1650 mg/L NH_4NO_3 and sucrose as the carbon source, and enriched MS medium containing 250 mg/L NH_4NO_3 and maltose as a carbon source. With second medium, regeneration of all cultivars was found significantly better. Best responses were obtained from immature inflorescences which are 0.51 to 1.0 cm in length. According to results of best treatments, 16.1, 12.4, and 6.4 shoots per 10 explants were obtained in 3 different cultivars.

In 2000, Benkirane and co-workers obtained plant regeneration from fragments of immature inflorescences and coleoptiles of durum wheat via somatic embryogenesis. In their results, for inflorescence fragments, the highest frequencies of embryogenic callus formation (100% for most cultivars) and regeneration (100 % for two cultivars) were obtained from 0.5-1 cm long fragments.

In 2001, He and Lazzeri tested the regeneration capacity of scutellum and inflorescence explants of four genotypes of durum wheat and concluded that scutellum cultures gave higher frequencies of embryogenesis and plant regeneration than inflorescence cultures. The regeneration frequencies were found to be 97% to 100% for scutellum cultures with induction medium containing 2 mg/L picloram and 45-80% for inflorescence cultures with 4 mg/L picloram in induction medium.

One of the studies that investigate the embryogenic potentials of Turkish wheat cultivars from immature embryo and inflorescence explants was conducted by İpek Zeynep Durusu (2001). In this study, the regeneration potential of eight Turkish wheat cultivars was compared and immature inflorescences were found to have higher regeneration capacities than immature embryos and cultivars Doğankent, Seyhan and Yüreğir was identified as the genotypes with highest regeneration potentials.

1.4. Transformation of Wheat

Different transformation methods which are being used in other plant systems have been attempted to adapt to wheat with varying degrees of success. To date, only biolistics (particle bombardment), electroporation, and *Agrobacterium*-mediated transformation methods have been reported to produce fertile transgenic wheat plants with stable integration of the transgenes (Janakiraman *et al.,* 2001).

1.4.1. Transformation of wheat protoplasts

Protoplasts are plant cells which do not possess cell wall. Due to the absence of cell walls, direct delivery of foreign gene is thought to be easier. Thus, protoplasts were extensively utilized in the initial attempts of wheat transformation. Their use as explants for direct gene transfer was facilitated by the development of suitable techniques for isolation of protoplasts from different tissues. The first report of direct gene transfer in wheat protoplasts was by Lorz and co-workers (1985) from cultured cells *Triticum monococcum* by PEG-mediated uptake. The physiological age of the developing grain and the physiological state of the isolated protoplasts affected the level of transient gene expression. Some other significant factors affecting the transient activity of the reporter gene constructs were found to be the presence of the divalent cation Mg^{2+} with PEG, time lapse after DNA uptake, pre-

culture medium and the regulatory elements of the vector construct. These reports yielded low transformation efficiencies in the order of 0.005%. The low efficiencies along with the difficulties arose by the regeneration of plants from protoplasts forced researchers to utilize alternative target cells or tissues with known regenerating capacities (Patnaik and Khurana, 2001). Accordingly, subsequent researches have focused more on embryogenic suspension cells and embryogenic callus cultures derived from scutellar tissue of mature and immature embryos.

1.4.2. Electroporation of wheat protoplasts and organized tissues

Electroporation uses high voltage electrical pulses to create transient pores in the cell membrane and, in this way, the uptake of macromolecules like DNA is facilitated from a surrounding buffer solution. Ou-Lee et al. (1986) reported the expression of the bacterial chloramphenicol acetyl transferase (*cat*) gene after electroporation of protoplasts of three important graminaceous plants; rice, sorghum and wheat. The survival percentage of protoplasts after electroporation depended largely on the tissue of origin. Stable transformation by electroporation (Zhou *et al.,* 1993) was reported by the use of the plasmid pBARGUS into protoplasts isolated from cell suspension initiated from an anther-derived callus. Stable transformation of protoplasts was also reported by He and co-workers (1994) by electroporation employing the *bar* gene as the selectable marker with a transient expression of *gus* gene at a frequency of $1x\ 10^{-5}$. Electroporation of protoplasts considerably improved the gene transfer efficiency as compared to that achieved with PEG mediated approach.

The utilization of electroporation technique was extended to organized tissues by the study of Kloti and co-workers (1993) which reported the transfer of reporter genes (anthocyanin regulatory gene and the *gus* gene) by elecroporating zygotic wheat embryos. Introduction of *gus* gene was also reported by electroporating

organized tissue from slow-growing, embryogenic calli (Zaghmout, 1994). In 1998, He and Lazzeri reported the optimized conditions such as electroporation voltage, the pulse length, volume of the electroporation buffer, osmoticum of the buffer and medium etc. for tissue electroporation of wheat scutellum cells and Tritordeum inflorescences. They suggest that their optimized protocol has a negligible effect on the regeneration of the plants. Recently, the production of fertile transgenic wheat plants by this method from intact immature embryos was reported by Sorokin and colleagues (2000) at a transformation frequency of 0.4%.

The efficiency of electroporation depends on a combination of factors which includes pre-treatments of the recipient tissue and the culture conditions. Patnaik and Khurana (2001) supports that due to its technical simplicity and low cost, tissue electroporation may become as a routine transformation method (Patnaik and Khurana, 2001). However, although the technique works in all major cereal species, the transformation efficiency is not sufficiently high especially for wheat. One disadvantage of electroporation could be that the target tissue preparation is critical and the amount of DNA delivery into the target cells is less than that of particle bombardment (Rakszegi *et al.*, 2001).

1.4.3. Transformation of wheat by microprojectile bombardment

In order to overcome the biological limitations of *Agrobacterium* and difficulties associated with regeneration from protoplasts, the idea of introducing DNA into cells by physical means was developed and extended to the invention of microprojectile bombardment. Biolistic particle delivery is a mechanical method of transformation that uses gas pressure to introduce DNA-coated microcarriers into intact plant cells, tissues, and organs (Rakszegi *et al.*, 2001). It was developed by Sanford in 1987 (Sanford *et al.*, 1987). The firstly invented types was using gun powder explosion instead of gas pressure for the acceleration of microcarriers. DNA is removed from the microcarriers and ultimately inserts itself into the cell's

(usually nuclear) genome. Usually, the insertion events are random and characterized by multiple copies and a certain degree of rearrangements (Jenes *et al*., 1993).

After the helium-driven gun (Kikkert, 1993), microprojectile bombardment method became a widely-used, standard method. The first commercial GM cereal varieties were produced by microprojectile bombardment method. In wheat, efficiency of 9.7 % (Zhang et al., 2000) was reported when immature embryos were transformed.

Initial studies on particle bombardment as a gene delivery method achieved transient expression of *gus* gene following bombardment of cell suspensions of *T. monococcum* by Wang and co-workers (Wang *et al*., 1988). Wheat is utilized as a target tissue for the first time by Londsdale and co-workers (1990) who transformed mature wheat embryos and showed transient expression of the *uid*A gene. In 1991, Vasil and colleagues (Vasil *et al.,* 1991) obtained stably transformed callus lines that expressed all the marker genes tested (*gus*, *nptII* and *EPSPS*). In 1992, Vasil's group (Vasil *et al.,* 1992) obtained first transgenic plants by particle bombardment of plasmid vector pBARGUS into long-term regenerable embryogenic callus and this report is considered to be a milestone in transgenic wheat research.

The effects of various bombardment parameters like amount of plasmid DNA, spermidine concentration, acceleration and vacuum pressure, osmotic pretreatment of target tissues on gene delivery into wheat tissues have been investigated in detail in the study of Rasco-Gaunt and co-workers (Rasco-Gaunt *et al.*, 1999) who transformed ten European wheat varieties at different efficiencies ranging from 1 to 17 %. In later studies, it has been shown that the transgenes introduced by biolistic approach display a considerable degree of stability in integration and expression in subsequent generations (Altpeter *et al.*, 1996; Srivastava *et al.*, 1996).

There are two major requirements for efficient transformation. The first is the efficient delivery of particles into large numbers of target cells. The second is a high level of division and regeneration in the targeted cells. In cereals two types of target systems are used for bombardment. In the first method, primary explants are bombarded immediately or soon after isolation (Rakszegi *et al.,* 2001). The cells are induced to become embryogenic and regenerate (Barro *et al.*, 1997). The second technique uses with pre-established proliferating embryogenic cultures. Bombardment is followed by further proliferation and regeneration (Vasil *et al.*, 1992). Modifications to culture procedures, such as the plasmolysis of the tissues prior to bombardment or culture on high-osmotic media (Finer et al., 1999), help the targeted tissues to tolerate the damage of the bombardment process.

1.4.4. *Agrobacterium*-mediated transformation of wheat

Agrobacterium tumefaciens mediated transformation is another possible method for cereal transformation. *Agrobacterium tumefaciens*, a soil bacterium, can genetically transform plant cells with a segment of DNA from tumour-inducing plasmid (Ti plasmid) with the resultant production of a crown-gall, which is a plant tumour. Crown gall is a disease that causes considerable damage to perennial crops. This technique is widely used successfully for most of the dicotyledonous plants (Rakszegi *et al.,* 2001).

The preferred method of cereal transformation has been the direct transfer of DNA by biolistic devices, mainly because *Agrobacterium* mediated gene transfer was believed to be limited only to dicots (Thomashow *et al.,* 1980). The *Agrobacterium*-mediated transformation system offers several advantages including ;

i. highly efficient defined insertion of a discrete segment of DNA into the recipient genome with fewer rearrangements and in small number of copies,
ii. high co-expression of multiple introduced genes,
iii. simplicity and low cost.

These advantages allow production of plants with simple integration pattern along with high frequency of stable genomic integration and single-low copy number of the intact transgene (Rakszegi *et al.,* 2001; Janakiraman *et al.,* 2001; Sahrawat *et al.*, 2003).

For several years, cereals were classified as non-hosts for *Agrobacterium*, as they were not infected *in vivo* or *in vitro*. Recent investigations have shown that *Agrobacterium* can attach to cereal cells, that these cells produce factors that induce *Agrobacterium* virulence genes (Hoisington *et al.*, 2002). In 1980-90s, researchers started to re-evaluate the potential of *Agrobacterium* as a transformation vector. Initial studies on *Agrobacterium* mediated transformation of wheat is performed via Agroinfection and it was demonstrated for the first time that the soil bacterium can interact with the cells of a monocot plant and transfer DNA in a limited manner (Woolston *et al.*, 1988; Dale *et al.*, 1989). Agroinfection is the introduction of viral DNA residing between the T-DNA border sequences of *Agrobacterium* into plant cells and provide a suitable system to monitor DNA delivery into target explants by detection of disease symptoms (Patnaik and Khurana, 2001).

In 1990, Hess and co-workers reported pipetting *Agrobacterium tumefaciens* into spikelets to introduce kanamycin resistance. Although the trait was found to be inherited, supportive molecular evidence was lacking. The method can be considered as first report of floral dip method.

Mooney and colleagues (1991) reported the first transformation of wheat embryo cells co-cultivated with *Agrobacterium tumefaciens* and for the first time demonstrated that wounding is not necessary for adherence of bacteria to explants of wheat. Mooney suggested that the increase in adherence of bacteria at the wound site caused by mechanical and enzymatic treatments. Later, in 1992, Chen and Dale reported a higher frequency of infection in exposed apical meristems of dry wheat seeds as compared to intact seeds.

In 1993, Chan and colleagues produced transgenic rice plants by inoculating immature embryos with *Agrobacterium tumefaciens*. In 1994, Hiei and co-workers produced fertile transgenic rice plants. In the following years, efficient methods for *Agrobacterium* mediated transformation of rice and maize was reported (Rashid *et al.*, 1996; Ishida *et al.*, 1996; respectively). Although they utilize super binary vectors, their studies contributed to better understanding of various parameters required for successful transformation of cereals via *Agrobacterium*. In genetic transformation of cereals, another break through was reported by Tingay and co-workers (1997) who transformed barley embryos successfully by the utilization of a non-supervirulent *Agrobacterium* strain. Their success was achieved by the incorporation of phenolic compound acetosyringone, which is known to induce expression of virulence genes located on Ti-plasmid, into the co-cultivation medium.

First report of stable transformation of wheat by *Agrobacterium* mediated transformation released with the study of Cheng and co-workers in 1997. They also showed the successful transmission of the transgene to the next generation. This study developed a system for the production of transgenic plants within a total time of 2.5 to 3 months by co-cultivating freshly isolated immature embryos, precultured immature embryos and embryogenic calli. Approximately 35% of the transgenic plants received a single copy of the transgene and one to five copies of the transgene were integrated into the wheat genome without rearrangement. The authors also pointed out the importance of the presence of acetosyringone in the

inoculation and co-cultivation media and also the presence of surfactants in the inoculation medium (Cheng *et al.*, 1997).

In 1998, McCormac and colleagues reported transformation of wheat via inoculation of immature embryos with both *A. tumefaciens* and *A. rhizogenes* and demonstrated that anthocyanin-biosynthesis regulatory genes can be used as a visual marker and as a method for studying the localized transformation events. The localization of the transformed cells revealed a non-random distribution throughout each embryo and callus piece.

Also in 1998, Guo and co-workers investigated various factors and reported that acetosyringone and *Agrobacterium* strain were vital for achieving high frequency of transient GUS expression in transformed tissue of wheat. Xia and co-workers, in 1999, further refined the protocol employed by Guo and produced fertile transgenic wheat plants at the rate of 3.7-5.9 % by using *A. tumefaciens* strain AGLI.

In 2001, Weir and colleagues reported *Agrobacterium*-mediated transformation of four varieties of wheat using GFP (green fluorescent protein) as visual marker and they recovered transgenic wheat plants at a transformation frequency of 1.8 % (Weir *et al.*, 2001). In the same year, *Agrobacterium*-mediated transformation of immature inflorescence tissue was reported by Amoah and colleagues (Amoah *et al.*, 2001). In this study, transformation efficiency was measured by GUS activity and optimal T-DNA delivery was achieved in explants precultured for 21 days and, also, vacuum infiltration and sonication were found to increase the frequency of GUS expression.

In 2003, Khanna and Daggard produced fertile transgenic wheat plants from immature embryo derived calli of spring wheat. In this report, authors demonstrated the superiority of super binary vector over binary vector and the

positive effect of exogenously applied polyamine spermidine in the recovery of transformants.

Besides successful reports on transforming wheat by using *Agrobacterium,* the induced cellular necrosis upon pathogen infection still stays unsolved as the major drawback in routine application of *Agrobacterium* for genetic transformation of wheat. After pathogen invasion, hypersensitivity response is triggered and induced cellular necrosis is observed as a typical resistance against further infection of the pathogen. In 2002, Parrott and co-workers have reported that immature embryos inoculated with *Agrobacterium* increased production of H_2O_2, browned and displayed altered cell wall composition and higher level of cellular necrosis leading to cell death (Parrott *et al.*, 2002). They also stated that the reduction of the O_2 tension from 7.4 to 2.1 mM during interaction of *Agrobacterium* with tissues significantly reduced the extent of embryo and root cell death.

In near future, it can be expected that *Agrobacterium* will be employed as a reliable, efficient and economical vector for the introduction of agronomically important genes provided that *Agrobacterium* associated cellular necrosis can be eliminated by the utilization antioxidants during co-culture of tissues with *Agrobacterium*, or reduction of oxygen during the plant-bacterium interaction.

1.4.5. Agronomically important genes transferred to wheat

Obtaining successful stable integration of transgenes into wheat genome has opened doors for incorporation of agronomically important genes in order to improve wheat quality and yield. These studies are mainly focused on resistance against biotic and abiotic stresses. Besides these, quality improvement and male sterility are also targets of wheat improvement. Agronomically important genes incorporated into wheat via particle bombardment is demonstrated in Table 1.4.

Table 1.4. Agronomically important genes transferred into wheat

(Adopted from Sahrawat *et al.*,2002).

Target tissue	Source of the gene	Gene	Selectable marker	Phenotype	References
IE	Barley yellow mosaic virus	Coat protein (cp)	*bar*	No data on phenotype	Karunarante *et al.*, 1996
IE	*T. aestivum* L.	High molecular weight glutenin subunit (1Ax1)	*bar*	Accumulation of glutenin subunit 1Ax1	Altpeter *et al.*, 1996
IE	*T. aestivum* L.	High molecular weight glutenin hybrid subunits (Dy10:Dx5)	*bar*	Accumulation of hybrid glutenin subunit	Blechl and Anderson, 1996
EC	*Bacillus amyloliguef aciens*	Barnase	*bar*	Nuclear male sterility	Sivamani *et al.*, 2000
IE	*T. aestivum* L.	High molecular weight glutenin subunits Dx5, 1Ax1	*bar*	Increased dough elasticity	Barro *et al.*, 1997
IE	*Vitis vinifera*	Stilbene synthase (Vst1)	*pat*	No data on resitance to fungus diseases	Leckband and Lörz, 1998
IE	*T. aestivum* L.	High molecular weight glutenin hybrid subunits (Dy10:Dx5)	*bar*	Accumulation of hybrid glutenin subunit	Blechl *et al.*, 1998
IE	*Oryza sativa*	Rice chitinase	*bar*	No data on phenotype	Chen *et al.*, 1998
EC	*Hordeum vulgare* L.	Class II chitinase (*chiII*)	*bar*	Resistance to fungus (*E. graminis*)	Bliffeld *et al.*, 1999

Table 1.4. Continued

IE	*O. sativa*	Thaumatin-like protein (*tlp*), chitinase (*chi11*)	*bar , hpt*	Resistance to fungus (*F. graminearum*)	Chen *et al.*, 1999
IE	*Zea mays*	Transposase (Ac)	*bar*	Synthesis of an active transposase protein in transgenic Ac line	Stöger *et al.*, 2000
IE	*T. aestivum* L.	High molecular weight glutenin subunit (1Dx5)	*Increased dough strength*		Rooke *et al.*, 1999
IE	*T. aestivum* L.	High molecular weight glutenin subunits (1Axx1, 1Dx5)	*bar*	Increased dough strength and stability	He *et al.*, 1999
IE	*H. vulgare* L.	Trypsin inhibitor (CMe)	*bar*	Resistance to angoumois grain moth (*S. cerealella*)	Altpeter *et al.*, 1999
IE	*Galanthus-nivalis agglutinin* (GNA)	Agglutinin (*gna*)	*bar*	Decreased fecundity of aphids (*Sitobin avanae*)	Stöger *et al.*, 1999
IE	*H. vulgare* L.	Chimeric stilbene synthase gene (*sts*)	*bar*	Production of phytoalexin resveratrol, no data on resistance to fungus diseases	Fettig and Hess, 1999

Table 1.4. Continued

IE	Wheat streak mosaic virus	Replicase gene (*NIb*)	*bar*	Resistance to wheat steak mosaic virus (WSMV)	Sivamani *et al.*, 2000
IE	*H. vulgare* L.	HVA1	*bar*	Improved biomass productivity and water use efficiency	Sivamani *et al.*, 2000
IE	Monoclonal antibody	T84.66 Single chain Fv antibody (ScFvT84.66)	*bar , hpt*	Production of functional recombinant antibody in the leaves	Stöger *et al.*, 2000
EC	*U. maydis* infecting virus	Antifungal protein (KP4)	*bar*	Resistance against stinking smut	Clausen *et al.*, 2000
IE	*A. niger*	Phytase-encoding gene (*PhyA*)	*bar*	Accumulation of phytage in transgenic seeds	Brinch-Pedersen *et al.*, 2000
IE	*H. vulgare* L.	Ribosome-inactivating protein (*RIP*)	*bar*	Moderate resistance to fungal pathogen *E. graminis*	Bieri *et al.*, 2000
IE	*Tritordeum*, tomato, oat	S-adenosyl methionine decarboxylase gene (SAMDC), arginine decarboxylase gene (ADC)	*bar*	No data on phenotype	Bieri *et al.*, 2000
IE	*T. aestivum* L.	High molecular weight glutenin subunits (1Ax1, 1Dx5)		Flours with lower mixing time, peak resistance and sedimentation volumes	Alvarez *et al.*, 2001
IE	Bacterial ribonulease III, wheat streak mosaic virus	Bacterial ribonulease III (rnc70), coat protein (cp)	*bar*	No data on phenotype	Zhang *et al.*, 2001

Table 1.4. Continued

IE	*A. giganteus*, *H. vulgare*	Antifungal protein afp from *A. giganteus* , a barley class II chitinase and rip I	*bar*	Inhanced fungal resistance	Oldach *et al.*, 2001
IE	*T. aestivum* L.	FKBP73 WFKBP77	*bar*	Alteration in grain weight and composition in transgenic seeds	Kurek *et al.*, 2002
IE	*T. aestivum* L.	High molecular weight glutenin subunits (1Ax1, 1Dx5)	*bar*	No data on phenotype	Barro *et al.*, 2002
IE	*T. aestivum* L. (soft wheat)	Protein puroindoline (PinB-D1a)	*bar*	Increased friabilin levels and decreased kernel hardness	Beecher *et al.*, 2002
EC	*F. sporotrichioides*	*Fusarium sporotrichioides* gene (FsTRI101)	*bar*	Increased resistance to FHB (*F. graminearum*)	Okubara *et al.*, 2002
IPS	*Vigna aconitifolia*	D1-pyrroline-5-carboxylate synthetase (P5CS)	*nptII*	Increased tolerance to salt	Sawahel *et al.*, 2002
IPS	Wheat streak mosaic virus	Coat protein gene (CP)	*bar*	Various degree of resistance to wheat streak mosaic virus	Sivamani *et al.*, 2002

Abbreviations: IE, immature embryos; EC, embryogenic callus; IPS, indirect pollen system (in this system Agrobacterium suspension is pipetted on spikelets just before anthesis); bar , phosphinothricin acetyl transferase; nptII , neomycin phosphotransferaseII; hpt , hygromycin phosphotransferase; Dy10, a high molecular weight glutenin subunit (HMW-GS) gene sequence.

The first report of an agronomically important trait transferred to wheat was published 1996 by the incorporation of the coat protein of barley yellow mosaic virus (Karunarante *et al.,* 1996), however, no data on the biological activity of the recombinant protein was demonstrated. In following years, several studies on disease resistant was reported. In 1998, Chen and co-workers introduced the rice class I chitinase gene (*chil*1) into wheat by particle bombardment; however expression in the progeny was silenced. In 1999, the rice *chil*1 and rice thaumatin like protein gene (*tlp*) construct were co-transformed in 'Bobwhite' wheat plants. *Tlp* gene expression was stable in three generations; upon bioassays, delayed infection of *Fusarium graminearum* was shown. In the same year, barley seed class II chitinase gene along with either a ribosome-inactivating protein (*rip*) gene or β-1,3-glucanase gene was introduced into Bobwhite wheat by particle bombardment by Bliffeld and colleagues with stable expression and resistance to powdery mildew. In 2000, Bieri and co-workers introduced a barley ribosome-inactivating protein (RIP30) into wheat mediated by particle bombardment and reported the expression of RIP30 in transgenic wheat plants which resulted in moderate protection against *E. graminis*.

Transgenic wheat plants expressing stilbene synthase gene (Leckband and Lörz, 1998), a mutant bacterial ribonuclease III gene (Zhang *et al.*, 2001), antifungal protein gene KP4 (Clausen *et al.*, 2000), a barley *tlp* antisense gene (Pellegrineschi *et al.*, 2001), barley trypsin inhibitor gene (*cme*) (Altpeter *et al.*, 1999), snowdrop lectin (*Galanthus nivalis* agglutinin) GNA gene (Stöger *et al.*, 1999) were reported along with their resistance against associated pathogens.

The genes encoding for late embryogenesis abundant (LEA) proteins have attracted researchers for engineering wheat against drought (Sahrawat, 2003). In 2000, the abscisic acid responsive barley gene (*HVA* 1) which belongs LEA gene family, was introduced into a spring wheat cultivar by particle bombardment by Sivamani and colleagues. Tested transgenic lines were found to show improvement in efficient water usage. Another study for obtaining stress resistant

transgenic wheat plants employed insertion of Aldose reductase gene, for which regeneration studies are still going on (Ertuğrul, 2002).

Wheat quality improvement studies are mainly focused on increasing gluten content in order to enhance baking quality. Blechl and Anderson (1996) and Altpeter and his group (1996) reported the over-expression of high molecular weight (HMW) glutenin genes in Bobwhite wheat. Both groups showed significant alteration in the overall dough properties of transgenic seeds. In 1997, Barro and co-workers transformed two near-isogenic wheat lines with genes for HMW subunits and improved dough elasticity. In 1999, He and co-workers modified the quality of pasta making durum wheat with the introduction of high molecular weight glutenin subunit genes into wheat genome.

High degree of male sterility is required in order to prevent self fertilization and gene escape. In 1997, De Block and co-workers engineered wheat with nuclear male sterility. They introduced *barnase* gene under the control of tapetum specific promoters from corn and rice into wheat and prevented normal pollen development as a consequence. Stable transgenic plants were male sterile.

Studies concerning transposon tagging and molecular pharming in wheat have also been reported (Takumi *et al.*, 1999; Stöger *et al.*, 2000; respectively). Stöger and co-workers have produced a medically important single chain recombinant antibody against the carcino-embryonic antigen (CEA) using a biolistic approach and this is the first report of exploiting transgenic wheat as a bioreactor.

1.5. Aim of the study

For the improvement of wheat cultivars, regeneration and transformation parameters for the desired explant should be optimized. In order to obtain Turkish wheat varieties with better characteristics, their behaviors in tissue culture and

during transformation procedure should be addressed. The main objective of this study was to optimize regeneration parameters from immature inflorescences of Turkish bread wheat cultivar Yüreğir which is known to have a high regeneration capacity. For these reasons, this study was focused on:

i. the determination of embryogenic capacity and regeneration potential of different regions of inflorescence tissue,
ii. the demonstration of the effect of dark incubation period on regeneration potential,
iii. the correlation of regeneration capacity with other parameters such as number of shoots, length of leaves, number of spikes, weight and number of seeds.

This study also aimed to establish an efficient *Agrobacterium* mediated transformation protocol for Yüreğir immature inflorescence tissue by monitoring transient expression of *uid*A gene. The determination of important parameters such as explant age, co-cultivation medium, effect of vacuum infiltration was also in the context of this study.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1 Plant material

In this study, immature inflorescence of spring bread wheat *Triticum aestivum* cultivar Yüreğir-89 was used. The seeds were obtained from Çukurova Agricultural Research Institute, Adana. Agricultural information on cultivar Yüreğir was given in Appendix A.

2.1.2. Chemicals

The chemicals used in this study were obtained from Sigma Chemical Company (N.Y., USA), Merck Chemical Company (Deisenhofen, Deutschland), and Duchefa (Haarlem, The Netherlands). All of the solutions were prepared by using distilled water.

2.1.3. Plant Tissue Culture Media

For regeneration studies, two different media were used. For the induction and maintenance of callus cultures, MS based media containing MS basal salts (Murashige and Skoog, 1962), 3 % sucrose and 2 mg/L 2,4-dichlorophenoxyacetic

acid (2,4-D) was utilized. For regeneration medium, half strength MS basal salts and 3 % sucrose were used. The composition of MS medium is given in Appendix B. The media were solidified with 0.28 % Phytagel® and their pH were adjusted to 5.7-5.8 with NaOH or HCl. The media were sterilized by autoclaving at 121°C for 20 minutes.

During transformation optimization studies, MMA medium was used for bacterial inoculation of plants and for rinsing after co-cultivation. It contains MS basal salts without MS vitamins, 10 mM MES, 2% sucrose and 200µM acetosyringone (pH= 5.6). Cefotaxim (500 mg/L) was added to MMA medium during rinsing process. During bacterial co-cultivation period, MMD medium which is supplemented with 200µM acetosyringone and 100 mg/L ascorbic acid was utilized. A modified version of MMD medium, namely MMM medium, was also used for co-cultivation purpose. Preconditioning of calli prior to transformation process was performed on MME medium. Ascorbic acid and antibiotics (Cefotaxim) were sterilized with 0.2 µm pore sized filters and added freshly to sterile medium. The compositions and usages of plant tissue culture media are given in Table 2.1.

2.1.4. Bacterial Strains and Plasmids

In transformation studies, *Agrobacterium tumefaciens* strain AGL I containing pAL154 and pAL156 was used. AGL I is resistant to carbenicillin and the selectable marker for pAL 154 is *nptI*. There is no selection for pAL154, since pAL156 is dependent on pAL154. pAL156 contains *GUS* and *bar* genes. The map of the plasmids is given in Appendix C. The *Agrobacterium* strain including the plasmids was donated by Matthew D.Perry and Wendy Harwood from John Innes Centre. The transfer agreement is given in Appendix D.

Table 2.1. The compositions and usages of plant tissue culture media.

Name of medium	Composition	Usage
MS_2	MS basal salts + 3 % sucrose + 2 mg / L 2,4-D + 0.28 % Phytagel pH = 5.8	Callus induction and maintenance
MS (1/2)	½ MS basal salts + 3 % sucrose + 0.28 % Phytagel pH = 5.8	Regeneration of wheat embryogenic calli
MMA	MS basal salts excluding vitamins + 10 mM MES + 2 % sucrose pH= 5.6	Bacterial inoculation of explants, elimination of *Agrobacterium* when supplemented with Cefotaxim (500 mg/L)
MMD	MS basal salts + 10 mM MES + 3 % sucrose + 1 mg/ L 2,4-D + 200 µM Acetosyringone + 100 mg /L Ascorbic acid + 0.28 % Phytagel pH= 5.6	Co-cultivation of wheat calli
MMM	MS basal salts + 10 mM MES + 3 % sucrose + 0.5 mg/ L 2,4-D + 200 µM Acetosyringone + 100 mg /L Ascorbic acid + 0.28 % Phytagel pH= 5.0	Co-cultivation of wheat calli
MME	MS basal salts + 3 % sucrose + 1 mg/ L 2,4-D + 100 mg /L Ascorbic acid + 0.28 % Phytagel pH= 5.6	Preconditioning of wheat calli prior to transformation

2.1.5. Bacterial Culture Media

For the growth of AGL I, YEB medium which contains nutrient broth, yeast extract, sucrose and magnesium sulphate (pH= 7.4) was used (Appendix E). The media was supplemented with Kanamycin (100 mg / L) and Carbenicillin (200 mg/L). In order to obtain single colonies, same medium with agar (1.5 %) as solidifying agent was utilized.

During transformation procedure, for the induction of *vir* genes YEB+MES medium containing Kanamycin (100 mg / L) and Carbenicillin (200 mg / L), and 20 μM Acetosyringone was utilized (Appendix E).

2.2 Methods

2.2.1 Tissue Culture Studies in Wheat

The effects of dark incubation period and explant region were tested for their differences in regeneration frequency.

2.2.1.1 Preparation of the plant material

The explants were obtained from wheat plants which were grown in greenhouse. Yüreğir seeds were planted in soil containing pots. Greenhouse conditions were set to 25°C and 16/8 day-night photoperiod. The plants were watered regularly.

2.2.1.2. Isolation of immature inflorescence

For obtaining immature inflorescences in the desired stage, plants were collected prior to the emergence of flag leaf and this period corresponds to 30-40 days for Yüreğir. At this time, the stems were harvested and their outer leaves were removed. 7-8 cm long segment of the stem, which contains the inflorescence in-between, was taken. This segment was surface sterilized by using 70 % (v/v) ethanol for 30 seconds and 20 % (v/v) sodium hypochlorite which contains a few drops of Tween-20 for 20 minutes consequently. Then these explants were rinsed with sterile distilled water in laminar air flow cabinet. Succeeding treatments were all performed under sterile conditions in laminar air flow cabinet.

The immature inflorescences were isolated from these sterilized wheat stem segments by the aid of scalpels fitted with blades and forceps under a stereomicroscope. The inflorescences were taken out of the inner layers of the explant by making vertical incisions. It is important to pay attention to prevent any damages on the inflorescence tissue. The isolated inflorescences were cut into three pieces and referred to as top, mid and base regions from top of the inflorescence through the bottom (Figure 2.1.).

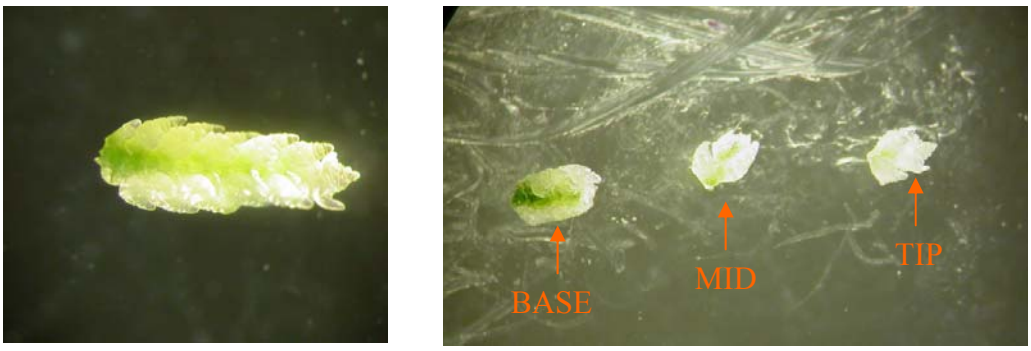


Figure 2.1. Immature inflorescence and its regions.

These pieces were put onto petri plates which contains callus induction medium (MS_2) without altering the regional order. About 21 explants were put onto each plate. After sealing with stretch film, the plates were incubated in the tissue culture room at 25°C under dark conditions.

2.2.1.3. Induction and maintenance of callus cultures

The induction and maintenance of callus cultures were conducted on MS_2 medium. Since the nutrient and plant growth regulator concentration in the medium diminishes with time, the calli were subcultured at 3-6 weeks intervals in order to provide maintenance of callus. During these transfers, top, mid, base regions were placed carefully for not to change their order.

2.2.1.4. Determination of callus growth rate

For the determination of callus growth rate, calli were transferred to a sterile Petri dish and the weight of calli present in a plate were measured in aseptic conditions. Afterwards, callus pieces were re-transferred to callus culture medium. This measurement was repeated every week for 8 weeks. At the third and sixth weeks, the medium was refreshed.

2.2.1.5. Determination of the effect of dark incubation period

For the determination of the effect of dark incubation period on regeneration success, an experiment set up was designed. After 6 weeks of dark incubation, each callus were cut into two. One piece was put onto callus maintenance medium and kept at dark as previously while other piece was put onto regeneration medium and transferred to light. The same procedure was applied to the pieces which were

kept in callus maintenance medium when they became 9 weeks old (after 3 weeks). The same procedure was repeated with the pieces which were kept at callus phase when they became 13 weeks old (after 4 weeks). At the 16th week, all of the calli were taken to regeneration. At the end, regenerated plantlets from callus cultures of 6 weeks, 9 weeks, 13 weeks and 16 weeks old were obtained (Figure 2.2).

2.2.1.6. Regeneration of wheat calli via somatic embryogenesis

After dark incubation period, calli were transferred onto Petri dishes containing regeneration medium (MS) in which strength of basal salts were reduced to half and 2,4-D was excluded. Root and shoot formation were observed accordingly. After 4 and 8 weeks data were collected as the number of shoot regenerating explants and number of root giving explants. For shoot regenerating explants, number of shoots per each explant was also recorded.

2.2.1.7. Growth of plants to maturity

After 4 or 8 weeks, plantlets were transferred to jars containing regeneration medium. When plantlets attain a considerable size (lasts nearly 4 weeks), they were transferred to soil. At this stage, the length of each tiller was measured.

2.2.1.8. Transfer of plantlets to soil

After growing in jars, plantlets were transferred to pots containing soil. The soil was previously autoclaved and cooled in order to prevent any contamination that might come through it.

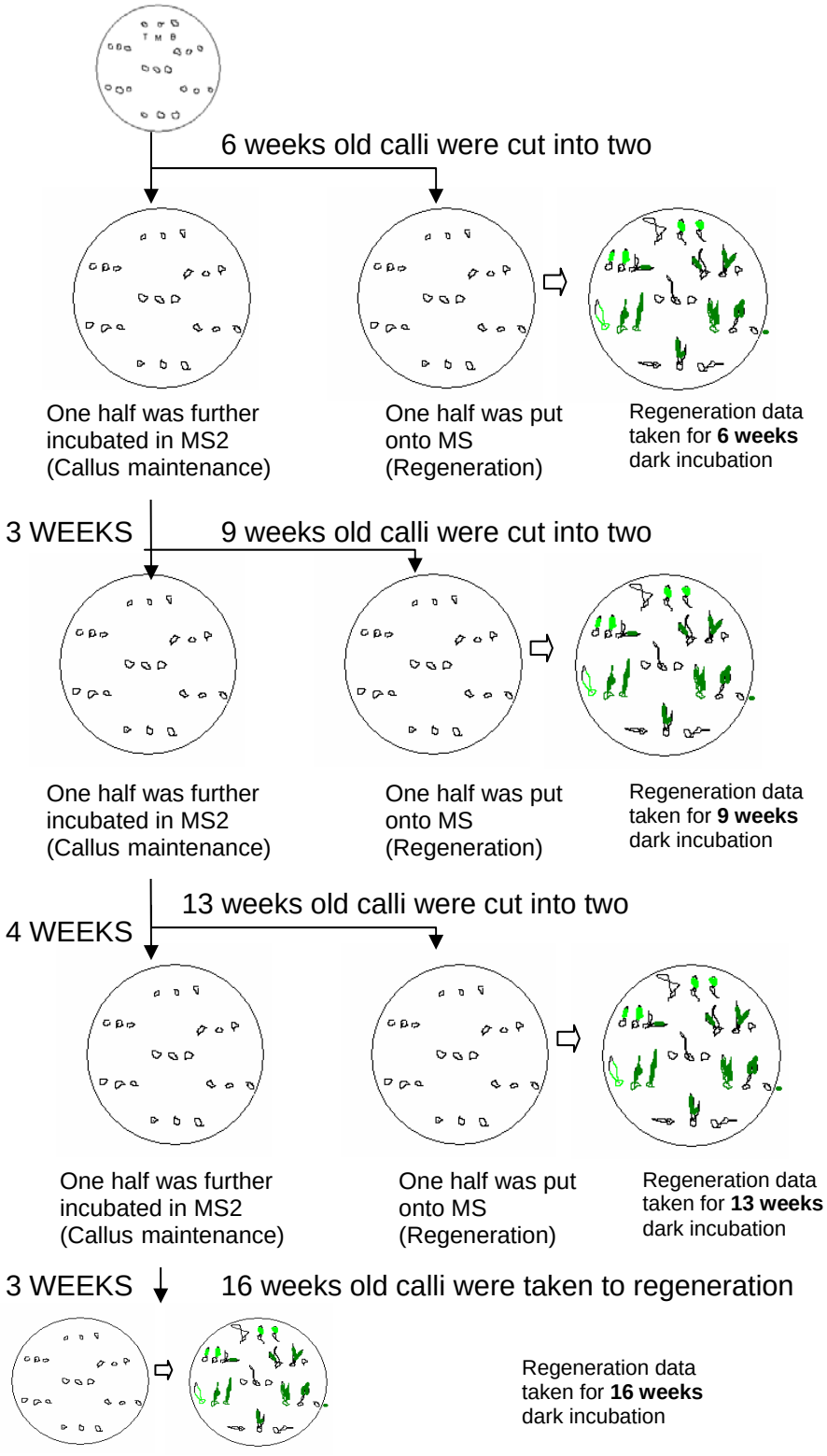


Figure 2.2. Schematic representation of experimental design for the determination of effect of dark incubation period on regeneration success.

2.2.1.9. Acclimization of plantlets to greenhouse conditions

While their transfer to soil, plants were covered with a plastic bag in order to maintain a humid environment so that the plants do not sense a great stress after *in vitro* conditions. In several day intervals, some openings were made on the plastic bag in order to make the plant to get used to greenhouse environment gradually.

2.2.1.10. Obtaining seeds

Plants which were acclimized to greenhouse environment were watered regularly. They were fertilized in two weeks intervals. They grown to maturity and gave seeds. When the plant became yellow and all seeds were mature, spikes were collected. The number of spikes for each plant, the number of seeds for each spike and the weight of each seed were recorded.

2.2.2 Transformation Studies in Wheat

Optimization of transformation of Yüreğir immature inflorescence-based calli with *Agrobacterium tumefaciens* AGLI was performed.

2.2.2.1. Preparation of immature inflorescence-based callus cultures

Immature inflorescences were collected, isolated and put onto callus induction medium as previously mentioned on section 2.2.1.2 and 2.2.1.3. The inflorescences were kept at MS_2 media under dark conditions at 25°C and transformation studies were carried out by using calli at different ages.

2.2.2.2. Growth of *Agrobacterium tumefaciens* AGL I

Agrobacterium tumefaciens strain AGL I were grown in liquid YEB medium supplemented with Kanamycin (100 mg / L) and Carbenicillin (200 mg/ L). Prior to transformation studies, single colonies were selected from YEB+agar culture and inoculated into 5 mL YEB cultures. Liquid bacterial cultures were incubated at 28°C with vigorous shaking (180 rpm) in Gallenkamp shaking incubator.

A liquid aliquot from single colony were stored at -80°C in 80 % glycerol for long term storage, or at -20°C in 50 % glycerol for short term storage.

2.2.2.3. *Agrobacterium*-mediated transformation of wheat inflorescence

The utilized *Agrobacterium*-mediated transformation protocol is adopted from Mahmoudian *et al*. (2002) and composed of several stages such as induction of *Agrobacterium vir* genes, inoculation of wheat calli with *Agrobacterium*, co-cultivation of the bacteria and plant and elimination of Agrobacterium.

2.2.2.3.1. Induction of *Agrobacterium vir* genes

Since *Agrobacterium tumefaciens* cannot infect *Triticum aestivum* in nature, the bacteria should be induced prior to transformation studies. According to the method developed by Kapila and co-workers (1997), when bacteria are in log phase, they were inoculated into YEB+MES medium which is supplemented with Kanamycin, Carbenicillin and 20µM Acetosyringone as the phenolic compound. Besides, the pH of the medium was adjusted to 5.6 for further induction of the bacteria. The growth of the bacteria was monitored by OD measurements with Shimadzu UV visible spectrophotometer (1240). When the optical density of the bacterial culture was reached to about 0.8-1.2, the bacteria were collected by

centrifugation at 1500 g, for 15 minutes. Then for actual activation of the bacterial *vir* genes, cells were resuspended in MMA medium which contains 200 μM acetosyringone until optical density becomes 2.2-2.4. The bacteria were incubated at MMA medium at 22°C for 1, 1.5, 2 hours in dark conditions.

2.2.2.3.2. Inoculation and co-cultivation of wheat calli with *Agrobacterium*

During incubation period in MMA medium, the bacteria becomes ready to infect plant cells. The wheat calli were suspended in bacterial suspension for 1 h. At the end of inoculation period, explants were taken out and blotted on sterile filter paper. Next, the explants were co-cultivated in MS_2, MMD or MMM medium for 2-4 days in dark conditions.

2.2.2.4. Vacuum infiltration of wheat calli with *Agrobacterium* suspension

In order to determine the effect of vacuum infiltration on transformation efficiency, the wheat calli were infiltrated with activated *Agrobacterium* suspension at -600 mmHg or -200 mmHg pressure during the bacterial induction period with occasional shaking. The vacuum infiltration equipment is demonstrated in Figure 2.3. At the end of the inoculation period, explants were blotted and transferred to co-cultivation medium.

2.2.2.5 Elimination of *Agrobacterium*

After co-cultivation period, calli were washed with MMA medium supplemented with 500 mg/L Cefotaxim for 40 minutes. Then explants were blotted and put onto MS_2 medium for 3-4 days in order to allow for the expression of transformed genes.

Figure 2.3. Vacuum infiltration equipment.

2.2.2.6. Histochemical GUS assay

Seven days after transformation, calli were stained with histochemical GUS assay, according to the method of Jefferson (1987), in order to monitor transient gene expression. For this assay, explants were put into 1mM chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) containing GUS substrate solution (Appendix F) in plastic tubes and they were vacuum infiltrated at -200 mmHg for 2-3 minutes for better absorption of the substrate solution by the plant tissue. Then, the explants were incubated at 37°C for 2 days. At the end of this incubation period, the calli were examined under microscope for number of blue spots or regions. For fixation of the color, the tissue was stored in GUS fixative solution (Appendix F).

2.3. Statistical analyses

All of the statistical analyses were carried out by using Minitab software. Analysis of variance (ANOVA) was used to investigate the relationship between a response variable and one or more independent variables. Besides ANOVA, Pearson product moment correlation coefficient was used to measure the degree of linear relationship between two variables.

CHAPTER III

RESULTS AND DISCUSSION

3.1. Regeneration studies

Callus induction and the effect of dark incubation period on regeneration capacity along with the effect of explant region on regeneration capacity will be demonstrated in this section.

3.1.1. Callus induction studies

Immature inflorescence of Yüreğir was used as the explant source. The inflorescences were isolated at the stage when they were 0.5-1.0 mm in length. This developmental period was found to respond best in forming callus as well as plantlets at very high frequency by several authors (Sharma et al., 1995; Caswell et al., 2000).

Callus induction was initiated from tip, mid, base regions of Yüreğir immature inflorescence in 2 mg/ L 2,4-D. Initially callus growth curve was determined without discriminating as tip, mid, base for monitoring the trend of growth in order to be able to compare it with following application of certain chemicals (Figure 3.1).

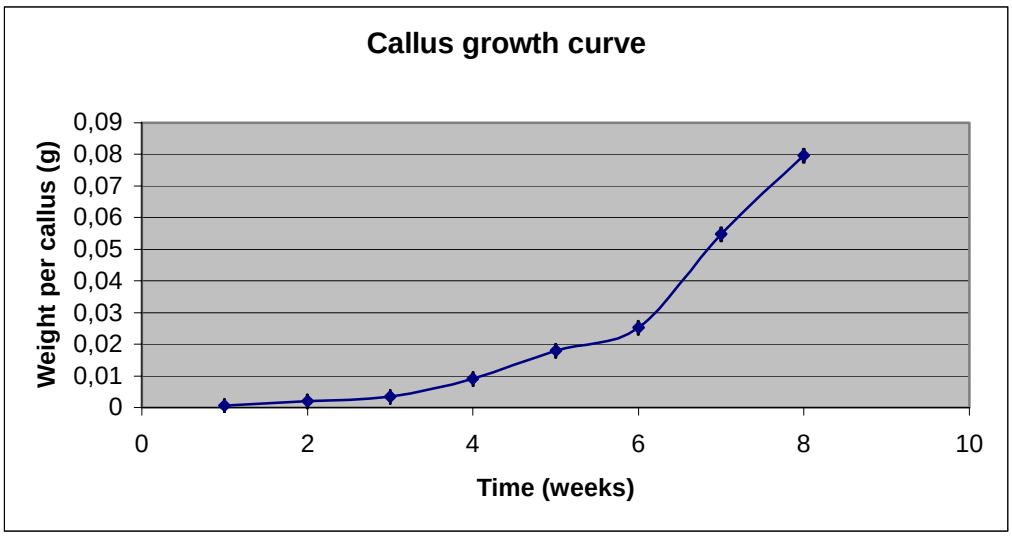


Figure 3.1. Callus growth curve obtained without discrimination as tip, mid, base.

In our study, with Yüreğir immature inflorescence, after 1-2 weeks of incubation at dark, callus formations became visible. Ozias-Atkins and Vasil have indicated in 1982 that the young inflorescence segments callused on MS_2 only after an extended lag period with respect to immature embryos. They also indicated that within a few days, the rachis and bases of the glumes became bright green but signs of callusing were not evident for two weeks. In this manner, our results seem to be consistent with the literature. The callus formation and appearance of callus in different incubation periods are given in Figure 3.2. All of the explants put onto callus induction medium responded and 100 % callus induction frequency was obtained. It was observed that there is no difference between different regions of the immature inflorescence in terms of callus induction.

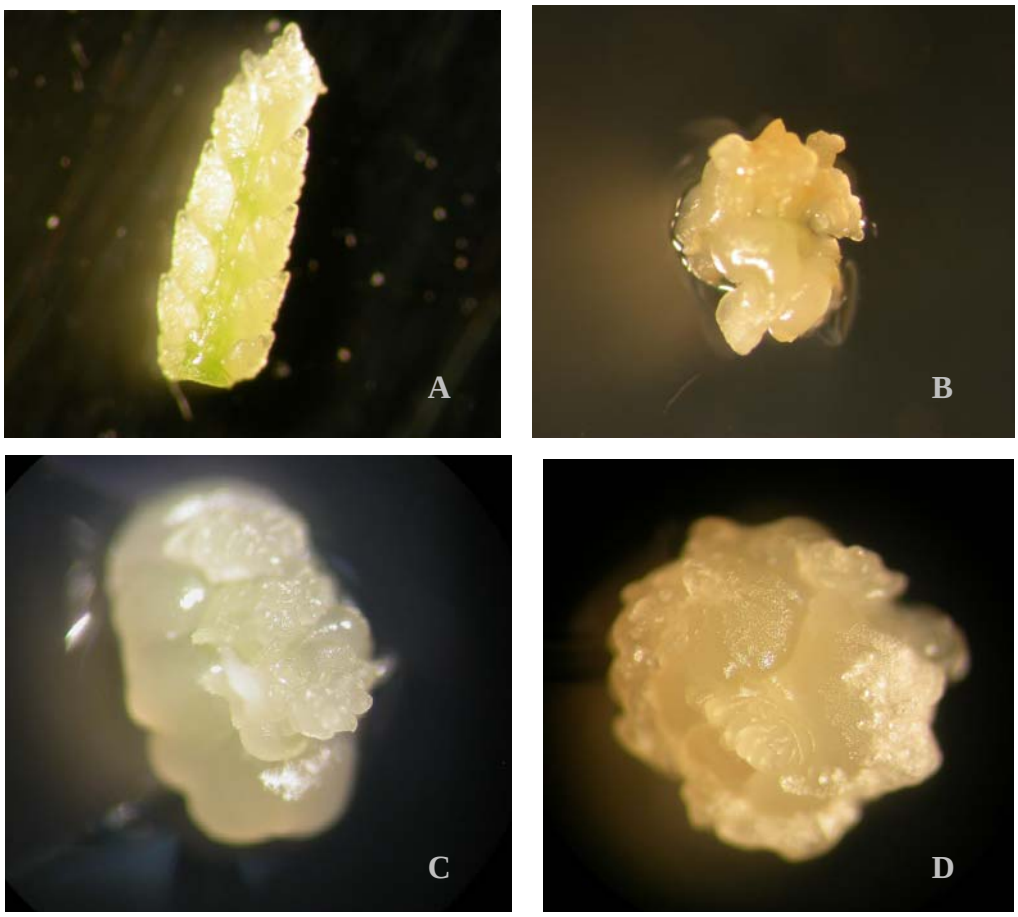


Figure 3.2. Callus development from Yüreğir immature inflorescence. A. Freshly isolated immature inflorescence. B. Yüreğir inflorescence after 9 days in callus induction medium. C. Proliferation of callus is visible in 14 day old callus (4x). D. Healthy embryogenic callus 24 days (5x).

3.1.2. Effect of explant region and dark incubation period on regeneration capacity

In order to understand the effect of dark incubation period on regeneration capacity, calli were incubated for 6 weeks, 9 weeks, 13 weeks and 16 weeks in dark. For each dark incubation period, there were 3 different types of calli which

are different in terms of their origin, namely coming from tip, mid, base regions of the immature inflorescence. During the isolation of immature inflorescences, each inflorescence could not be isolated in intact form and could not be cut into as tip, mid, base regions. The calli whose regional origin could not be determined were named as non-polar throughout the text.

At the end of the dark incubation period, half of each callus was taken to light on regeneration medium which is free of 2,4-D and contains MS salts in half strength. Germination of somatic embryos is usually induced on hormone-free culture medium or medium that contains low levels of an auxin or low levels of both an auxin and a cytokinin (Carman, 1995). It was also well documented by Vasil and Vasil (1981, 1982) that lowering of 2,4-D levels results in the organization of somatic embryos in wheat. The germination of somatic embryos was observed after transfer to light as illustrated in Figure 3.3.

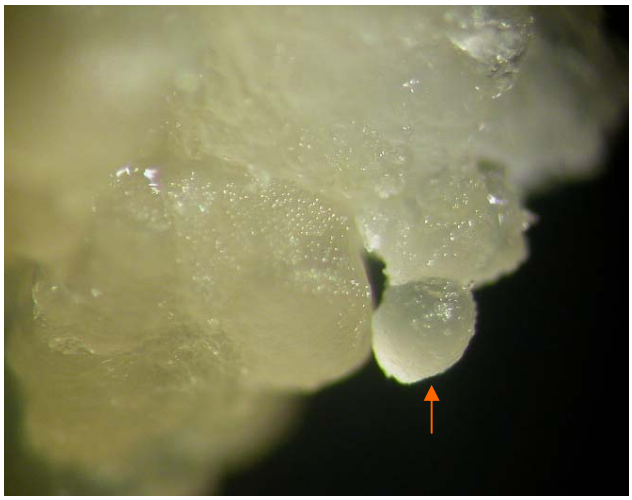


Figure 3.3. A globular structure on 40 days-old immature inflorescence-derived callus (magnification: 5x).

Ozias-Atkins and Vasil have observed formation of discreet nodules prior to germination of the somatic embryos in immature embryo derived calli (Ozias Atkins and Vasil, 1982). Later on, trichomes formed on the smooth nodular areas and they differentiate a leafy structure by the formation of a notch on the surface of a nodule, and finally the embryo-like structures were observed. In Yüreğir callus, at the end of 6 weeks period the similar globular structures were observed. This structures can be interpreted as those "notches" described by Vasil or can be considered as globular stage which is the first sequential stage of embryo formation in the somatic embryogenesis process (Figure 3.3, 3.4. and 3.5).

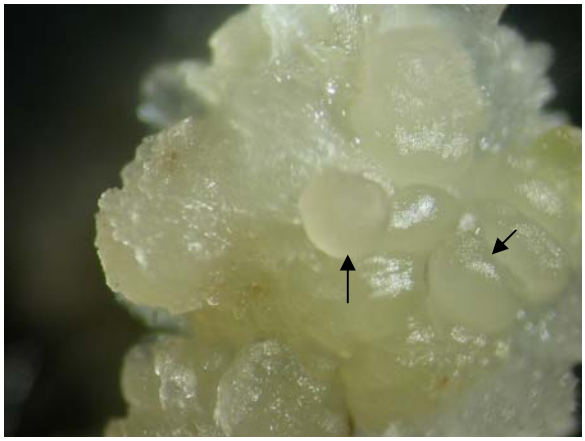


Figure 3.4. The structures shown by arrow might be globular and heart stages of the somatic embryoid development (6 weeks old, base).

Figure 3.5. The embryoid structures are also visible in callus which is taken onto regeneration medium (magnification: 6.5 x, mid).

In 5-10 days after transferred to regeneration medium, the emergence of green shoots and roots was observed. The appearance of the plantlets is given below in Figure 3.6 and 3.7.

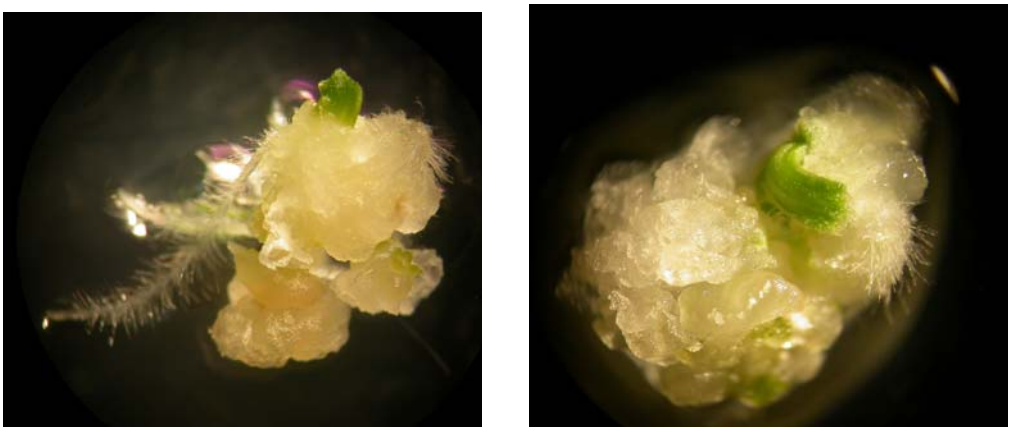


Figure 3.6. Regeneration from mid (magnification: 1.5x) and base (magnification: 2x) portions respectively, after 8 days on regeneration medium.

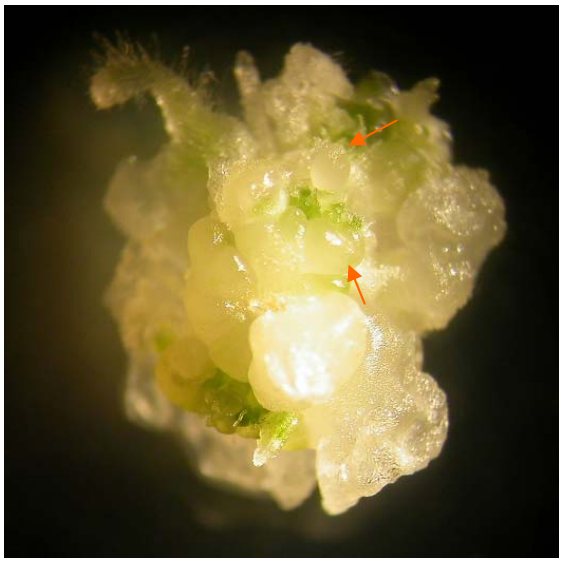


Figure 3.7. Embryoids which are still developing and regenerating from others can be observed 8 days after taken to regeneration medium.

The calli which are on Petri plates were incubated on regeneration medium for four weeks. At the end of four weeks, the regenerated plantlets were transferred to jars containing regeneration medium while non-regenerants were transferred to a fresh regeneration medium on Petri plates. The illustration of regenerated plantlets prior to being transferred to jars is given below (Figure 3.8).

Figure 3.8. Four weeks old plantlets prior to transfer to jars.

For the determination of regeneration capacity, number of shoot regenerating calli and number of root regenerating calli were recorded. The average shoot number per callus was also taken as data at the end of 4 weeks of incubation on regeneration medium (Table 3.1).

Since one of the characteristics of non-embryogenic calli is responding to regeneration only by rooting (Felföldi, 1992), rooting data were not used as an indicator of embryogenic capacity. In order to get embryogenic capacity of calli upon dark incubation period and explant origin, shooting frequency of each variable at the end of 4 weeks in regeneration medium was compared. Shooting frequency is defined as follows:

Shooting frequency = (Number of shoot regenerating calli / Total number of calli)

Table 3.1. Effect of dark incubation period and explant region on regeneration parameters. Data were collected at the end of 4 weeks of incubation on regeneration medium.

		Number of callus	Number of root regenerating calli	Number of shoot regenerating calli	Average shoot number per regenerating callus
6 WEEKS	Tip	37	37	21	3.41 ± 0.32
	Mid	37	37	16	3.00 ± 0.43
	Base	37	34	21	2.35 ± 0.24
	Non-polar	100	100	60	2.98 ± 0.19
	Total/Average	**211**	**208**	**118**	**2.95 ± 0.14**
9 WEEKS	Tip	41	41	18	3.20 ± 0.37
	Mid	31	31	18	2.67 ± 0.30
	Base	41	40	19	3.18 ± 0.33
	Non-polar	75	75	42	2.75 ± 0.20
	Total/Average	**188**	**187**	**97**	**2.90 ± 0.14**
13 WEEKS	Tip	32	30	5	3.0 ± 0.41
	Mid	32	32	7	2.71 ± 0.57
	Base	32	30	7	1.33 ± 0.33
	Non-polar	112	107	22	2.94 ± 0.36
	Total/Average	**208**	**199**	**41**	**2.63± 0.24**
16 WEEKS	Tip	6	5	1	1
	Mid	6	4	2	3.5 ± 1.5
	Base	13	6	2	4.5 ± 2.5
	Non-polar	90	86	12	3.14 ± 0.51
	Total/Average	**115**	**101**	**17**	**3.25 ± 0.52**

The number of explants for tip, mid and base regions of 16 weeks old calli was much lower than those of other dark incubation periods, thus, 16 weeks data were not utilized in comparisons of the effect of explant region.

When the shooting frequencies (number of shoot regenerating calli / total number of calli) of calli in terms of origin of the inflorescence region were compared, it was observed that there were no significant changes between tip, mid, base and non-polar origined callus in any of the dark incubation treatments (Figure 3.9.; $p = 0.329$ for 6 weeks, $p= 0.476$ for 9 weeks, $p= 0.915$ for 13 weeks in 95 % confidence interval). It can be interpreted that the endogenous plant hormones present in different regions of the immature inflorescence do not show variations which are sufficient enough to govern different developmental pathways and to cause different regeneration responses. It can also be concluded that any region of the immature inflorescence can be safely utilized in subsequent transformation studies and result in the same regeneration potential.

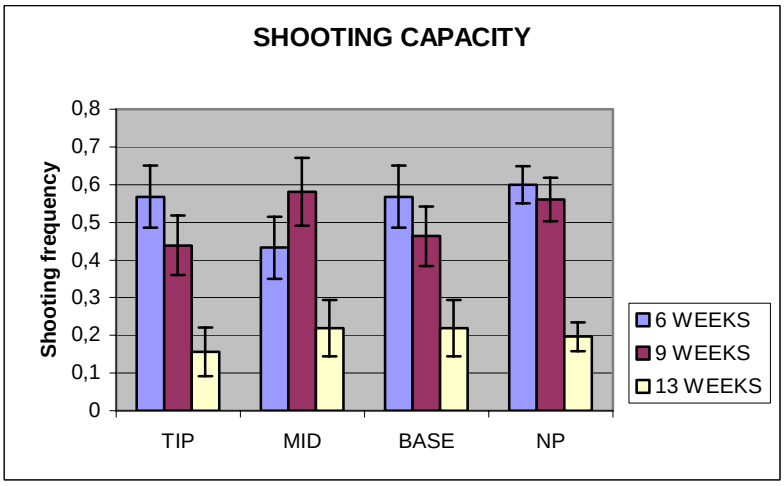


Figure 3.9. The comparison of shooting frequencies of tip, mid, base, and non-polar origined explants under different dark incubation durations.
(Shooting frequency = number of shoot regenerating calli / total number of calli)

Another comparison can be made between different dark incubation periods. Regardless of the origin of the explant; no significant difference was observed between 6 weeks and 9 weeks of dark incubation. However, 13 weeks dark incubation was significantly different from both 6 weeks and 9 weeks (Figure 3.9). Similar observation of decrease in regeneration potential with prolonged incubation in dark was observed in several studies. In a study conducted by Durusu (2000); the effect of dark incubation period on different Turkish wheat cultivars, including Yüreğir was well documented. Our findings are consistent with those results. The reason for the callus to "age" might be due to some changes in the cellular polyamine concentrations (Khanna and Daggard, 2001).

In some similar studies, researchers also calculate culture efficiency which is defined as the ratio of the number of shoot regenerating calli to the total number of explants at initial:
Culture efficiency = (Number of shoot forming calli / Total number of explants)

In our case, since callusing efficiency is 100 %, the shooting frequency is also equal to the culture efficiency.

The rooting capacities can also be compared in a similar fashion. The rooting capacities of explants are given as the ratio of root forming calli to total number of calli. When 13 weeks data were evaluated, the rooting capacity seemed to decrease with respect to 6 weeks and 9 weeks. For tip explant, the 13 weeks data was not significantly different from 6 weeks and 9 weeks with p= 0.084 while the difference in non-polar explants was found to be significant with a p value of 0.019 in 95 % confidence interval. The base was not significantly different from other treatments in every dark incubation period except 6 weeks dark treatment (p=0.002) (Figure 3.10).

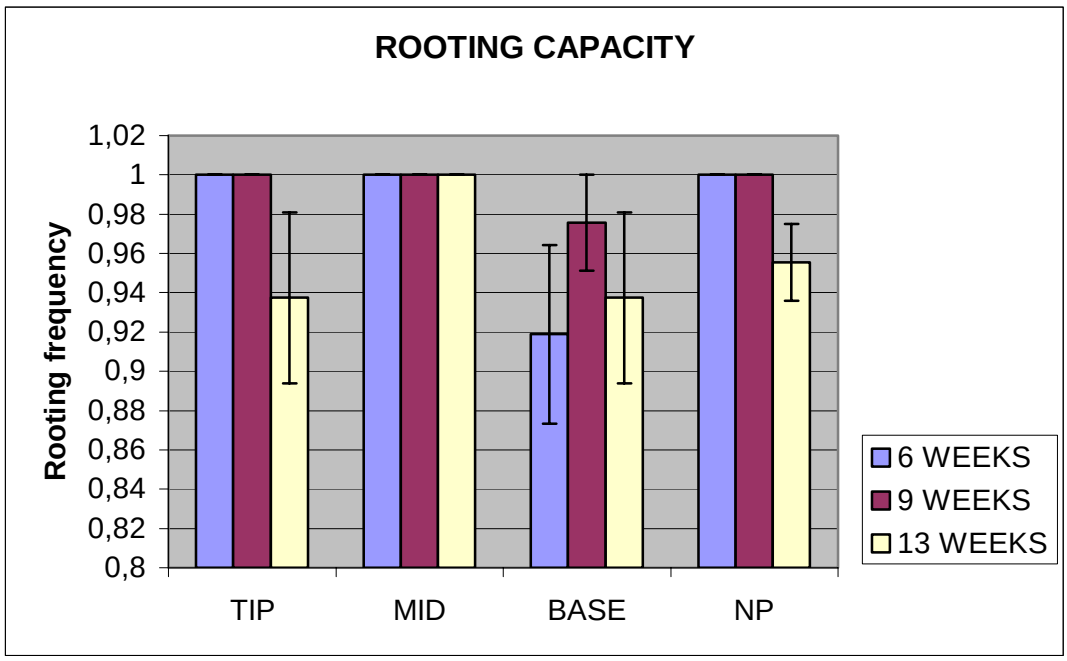


Figure 3.10. The comparison of root inductions.

The changes in root induction, in fact, do not make so much sense because some non-embryogenic calli can produce solely roots but not shoots (Vasil, 1987). Thus, root induction can not be correlated with embryogenic capacity. Our findings are also consistent with this fact.

In his report in 1987, Vasil has indicated the fact that many researchers described the formation of green, leafy structures prior to shoot development. However, from a developmental point of view leaves are formed only after the organization of a shoot meristem. According to Vasil, the so called green leaves reported earlier in immature embryo-derived calli were actually the enlarged scutella which became green and leafy in precociously germinating somatic embryos. For inflorescence-derived calli, similar situation might be relevant. Especially in 13 weeks and 16 weeks dark treatments, instead of formation of leaves after 4 weeks in regeneration medium, some green regions were observed (Figure 3.11). However, those regions

have not given rise to shoots and the shooting efficiency of prolonged dark incubation was found lower.

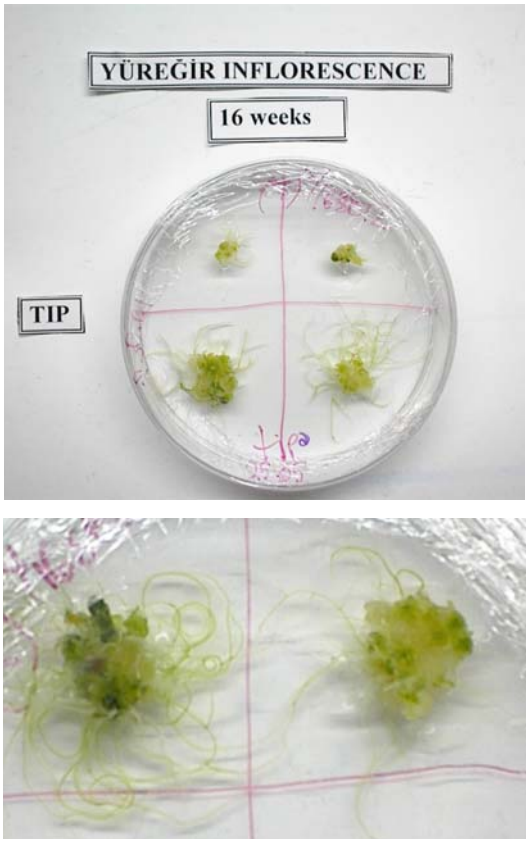


Figure 3.11. The formation of green regions which do not give rise to shoots shown in 16 weeks treated calli, with a closer view.

Since all of the calli taken onto regeneration medium do not produce shoots with the same rate, the differences in shoot number might be different in explants which regenerated earlier. Also in long term, shoot number can be an important parameter which contributes to grain yield. As He and Lazzeri stated in 2001, for the production of sufficient seeds in transgenic cereals, it is desirable to obtain

multiple tillers from primary transformant (T0) plants. In order to monitor this situation, the shoot numbers at the end of four weeks in regeneration medium was taken. The graph for average shoot number at the end of one month is given in Figure 3.12. The average number of shoots obtained from base region was found to be significantly different in 6 weeks, 9 weeks, and 13 weeks dark treatments. It might be due to the differences in the developmental period of the somatic embryos originated from base region. In fact, the strong inverse relationship between dark incubation duration and the germination of somatic embryos had been shown by the changes in shooting frequencies with dark incubation period and this situation was valid for every explant region (refer to Figure 3.9); however, the changes in average shoot number with respect to different dark incubation periods was found to be significant only in base region. Opposite to other explants with prolonged dark incubation periods not only the regeneration response but also the parameters which might be related with grain yield is affected in base region. For base region, nine weeks of dark incubation period seems to be the optimum duration at which maximum shoot number was obtained.

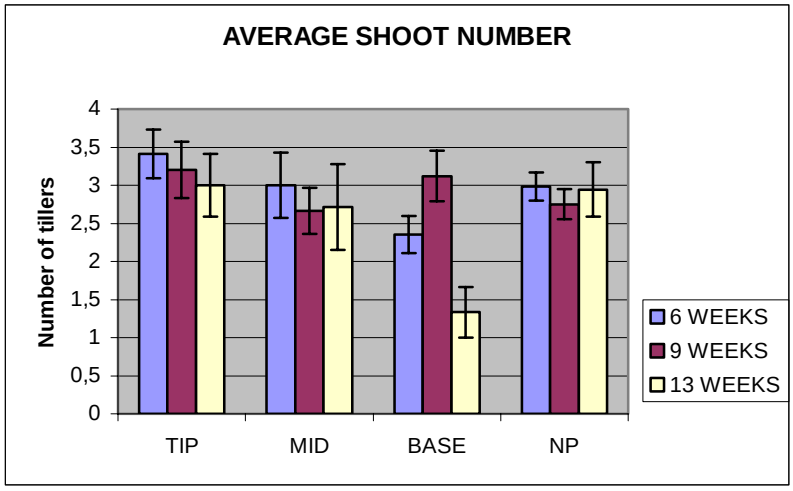


Figure 3.12. The comparison of average shoot number per regenerating callus at the end of 4 weeks.

It is also possible to pool data from tip, base, mid and non-polar in order to monitor solely the changes with different dark incubation periods. The changes in average shoot number without considering the explant region can be interpreted as such. When only the effect of dark incubation period was considered, no significant difference was observed in average shoot number as shown in Figure 3.13.

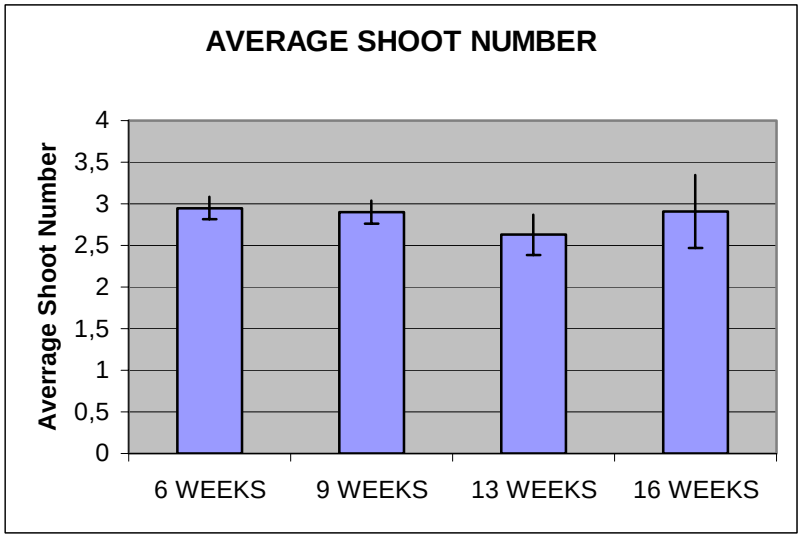


Figure 3.13. Average shoot number per regenerating callus by pooled data.

As far as we know, to date only Caswell and co-workers have scored the mean number of shoots produced by each regenerating inflorescence segment. They found the mean number of shoots produced by each regenerating explant of all four cultivars they have tested between 1.0 and 2.0 for all treatments. When it is compared with our results, Yüreğir inflorescence culture resulted in higher shoot numbers, around 3, at the end of 4 weeks light incubation period. It can be

concluded that cultivar Yüreğir is a good explant source and can result in high yield even at the end of *in vitro* culture.

The embryogenic capacities of explants, which are given as percentage of shoot induction, upon different dark incubation periods were given in Figure 3.14 by using pooled data. It was observed that there was no significant difference between root inductions of different dark incubation periods, whereas shooting potentials of 13 weeks and 16 weeks of dark incubation period were significantly lower than 6 weeks and 9 weeks of incubation (p=0).

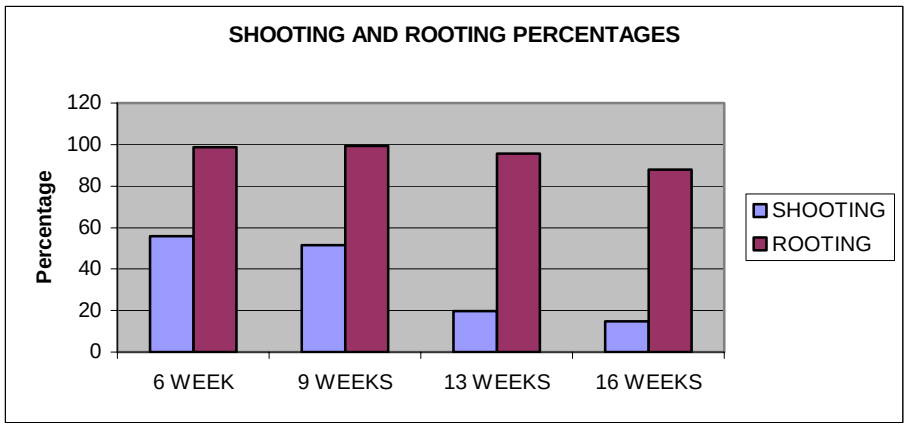


Figure 3.14. Effect of dark incubation period on shooting and rooting percentages.

According to these results, as time passes the germination potential of the somatic embryos decreases. In earlier times, there exists some precocious germination of somatic embryos as indicated in previous studies (Vasil, 1987). With prolonged incubation in 2,4-D containing medium, the germination of somatic embryos was suppressed and they lose the embryogenic potential over time. Still, 6 weeks and 9 weeks of dark incubation period seems to be optimum in long term transformation

studies. In order not to sacrifice from regeneration potential, the selection scheme of transformants before transferring to regeneration medium should not exceed up to 13 weeks.

3.1.3. Effect of prolonged incubation on regeneration medium

The regenerated plantlets were transferred to jars while non-regenerated ones were transferred to fresh regeneration medium on Petri plates and tracked for further 4 weeks. The shoot induction and root induction data were taken as second month data and cumulatively regeneration frequency of calli were represented in Table 3.2.

The reason for the increase in the regeneration capacity of callus might be due to the differences in the development rate of somatic embryos. In inflorescence tissue, the development of somatic embryos may still initiate during initial days following transfer to regeneration medium and thus result in different regeneration capacities upon time. Ultimately regeneration potential of Yüreğir inflorescence derived calli was reached to 72.0 %, 64.1%, 26.1 % and 24.3 % for 6, 9, 13, 16 weeks of dark incubation periods, respectively. The comparison of regeneration potentials of 4 weeks and 8 weeks of incubation on regeneration medium is given in Table 3.3.

Table 3.2. Effect on dark incubation period and explant region on regeneration parameters at the end of 8 weeks.

		Number of callus	Number of shoot regenerating calli	Number of root regenerating calli	Percent shoot induction	Percent root induction
6 WEEKS	Tip	36	26	35	72.2	97.2
	Mid	31	23	31	74.2	100
	Base	32	24	32	75	100
	Non-polar	101	71	100	70.3	99
	Cumulative	**200**	**144**	**198**	**72.0**	**99**
9 WEEKS	Tip	35	20	35	57.1	100
	Mid	29	20	29	69	100
	Base	36	23	36	63.9	100
	Non-polar	78	51	78	65.4	100
	Cumulative	**178**	**114**	**178**	**64.1**	**100**
13 WEEKS	Tip	28	5	27	17.9	96.4
	Mid	28	8	28	28.6	100
	Base	30	7	27	23.3	90
	Non-polar	94	27	91	28.7	96.8
	Cumulative	**180**	**47**	**173**	**26.1**	**96.1**
16 WEEKS	Tip	6	1	5	16.6	83.3
	Mid	6	2	4	33.3	66.6
	Base	13	2	11	15.4	84.6
	Non-polar	49	13	35	26.5	71.4
	Cumulative	**74**	**18**	**55**	**24.3**	**74.3**

Table 3.3. The increase in regeneration potential at the end of 8 weeks

	4 WEEKS DATA		8 WEEKS DATA	
	Percent shoot induction (%)	Percent root induction (%)	Percent shoot induction (%)	Percent root induction (%)
6 WEEKS	55.9	98.6	72.0	99
9 WEEKS	51.6	99.5	64.1	100
13 WEEKS	19.7	95.7	26.1	96.1
16 WEEKS	14.8	87.8	24.3	74.3

It is evident that longer regeneration time enhances the shooting response by about 16 % for 6 weeks, 12 % for 9 weeks, 6% for 13 weeks and 10 % for 16 weeks of dark incubation. For the regeneration of lately developed somatic embryos, prolonged time in regeneration medium seems to be very important. Its importance will be more apparent for transformation studies during which longer incubation periods for selection of transformants might be required either before or after taken to regeneration. The explants which pass through the transformation process are more delicate and may require more time for the development of somatic embryos. Thus, the explants should be kept in regeneration medium even longer than 8 weeks following transformation experiments.

The regeneration frequency of Yüreğir immature inflorescence tissue is pretty high when compared to other studies. In the report of Redway and co-workers (1990), immature embryos were found more suitable for regeneration. However, they have taken the calli to regeneration after 4 weeks of dark incubation. As shown in previous sections, 4 weeks of dark incubation might not be sufficiently long for the development of somatic embryos in immature inflorescence-derived cultures whose growth is known to be slower than immature embryos. In literature, for

immature inflorescences the regeneration frequencies were found to range between 29 to 100 % (Maddock *et al.*, 1983), 45-80% (He and Lazzeri, 2001). For immature embryos, regeneration frequencies were found to range 12 % to 96% (Maddock *et al.*, 1986), 2% to 94% (Fennel *et al.*, 1996), and 60% to 100 % with overall mean 71.2% (Bomnineni and Jauhar, 1996). For mature embryos, the regeneration potential were found to range between 26.8 % to 97.8 % (Özgen *et al.*, 1996); with mean values as 39 % (Zale *et al.*, 2003), 70.4 % (Özgen *et al.*, 1996), 96.1 % (Özgen *et al.*, 1998). For Turkish cultivars including Yüreğir, immature inflorescences responded better than immature embryos (Durusu, 2001). The advantages of using inflorescences over immature embryos are that they require reduced growth space and save time. Also explant isolation procedures are less time consuming. Immature inflorescences are primary explants, which are less likely than cell callus cultures to produce abnormal plants (Barcelo *et al.*, 1994). Usually, the cultivars used in biotechnology and tissue culture studies do not posses superior agronomic or quality traits (Zale *et al.*, 2004). Yüreğir is one of the good cultivars of Turkey which is suitable for climatic conditions of Turkey. When these positive aspects are considered, Yüreğir immature inflorescence is suggested as a good candidate in transformation studies for improvement of Turkish wheat varieties.

3.1.4. Shoot Growth Characteristics

After their transfer to jars, plantlets were grown one more month and then they were acclimized to soil. The appearance of plants which were acclimized to greenhouse conditions is shown in Figure 3.15.

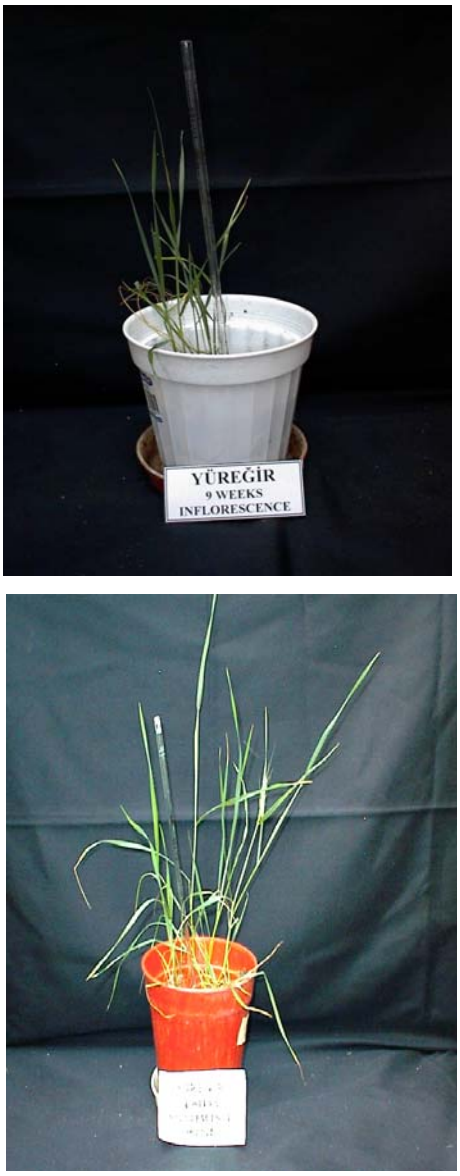


Figure 3.15. Plants transferred to soil and acclimized to greenhouse conditions (approximately 1 month after transfer to soil).

The plants which are grown in tissue culture conditions are more sensitive to environmental changes. Thus, the temperature and relative humidity were very important during the acclimatization process. Although an extensive care was shown to tissue culture origined plants, some of them were lost during

acclimatization. Throughout the experiments, the mortality rate did not reach to a high percentage. The sample size was smaller at later dates as a result of lower regeneration rates. The number of plants transferred to soil is presented in Table 3.4.

Table 3.4. Number of plants transferred to soil

		Number of plants transferred to soil
6 WEEKS	Tip	18
	Mid	10
	Base	9
	Non-polar	19
	Total	**56**
9 WEEKS	Tip	9
	Mid	16
	Base	16
	Non-polar	7
	Total	**48**
13 WEEKS	Tip	5
	Mid	5
	Base	7
	Non-polar	18
	Total	**35**
ALL OVER	**TOTAL**	**139**

During transfer to soil, which corresponds approximately 2 months after the calli were taken to regeneration, the number of shoots (tillers) originating from single callus and the length of the longest leaf from each tiller were recorded. Due to the differences in the germination rate of somatic embryos, the number of tillers during soil transfer might differ from the number recorded at the end of 4 weeks on the regeneration medium.

When the average shoot number during soil transfer was considered, 9 weeks dark incubated tip explant was significantly higher than tip 6 and tip 13 with a p value of 0.047; however, for mid, base and non-polar explants such a difference was not detected with respect to dark incubation periods. Average shoot number of tip 9 was also found to be significantly higher than mid 9 (p= 0.020). The comparison of treatments is shown in Figure 3.16.

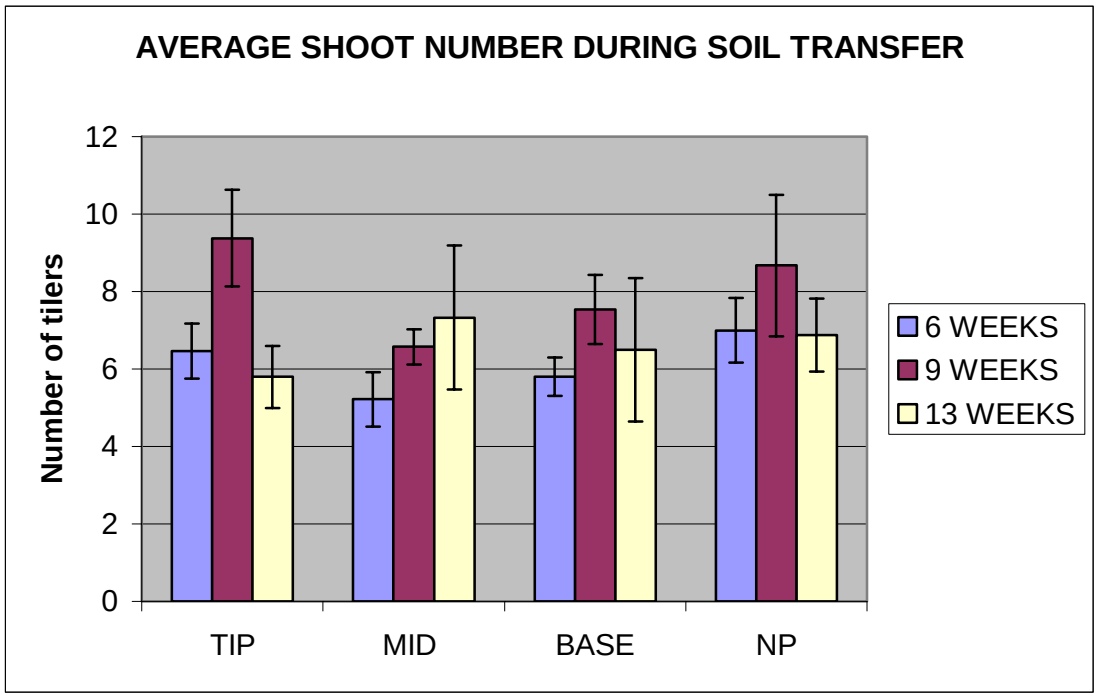


Figure 3.16. Average shoot number during soil transfer.

In order to detect differences coming only from different dark incubation periods, the data were pooled and compared accordingly. As shown in Figure 3.17, 9 weeks dark incubation period results in significantly higher number of tillers from 6 weeks with p = 0.026 but not from 13 weeks. The significance of 9 weeks dark incubation period might be due to the contribution of higher number of tip 9 explant.

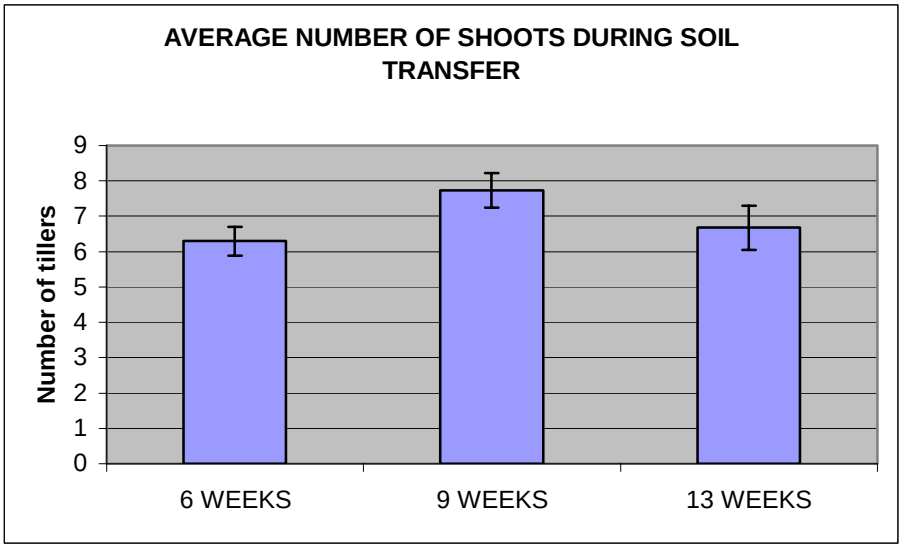


Figure 3.17. Average shoot number during soil transfer with pooled data.

Another parameter taken during soil transfer along with the number of tillers was the length of the longest leaf. Besides being an indicator of a healthy plant, the longer the leaves of a plantlet, the earlier the plant might have been regenerated from the callus. Thus, the length of the longest leaf at a specific time might be considered as a regeneration parameter. Total number of tillers and average length of the longest leaf is demonstrated in Table 3.5.

Table 3.5. The comparison of treatments in terms of number of tillers and average length of longest leaf.

		Number of tillers	Average length of longest leaf
6 WEEKS	Tip	109	19.0 ± 0.68
	Mid	48	20.4 ± 1.18
	Base	35	18.9 ± 1.43
	Non-polar	110	21.6 ± 0.67
	Total / Average	**303**	**20.1 ± 0,43**
9 WEEKS	Tip	77	20.0 ± 0.71
	Mid	106	20.0 ± 0.70
	Base	102	22.0 ± 0.75
	Non-polar	53	22.5 ± 1.02
	Total / Average	**338**	**21.0 ± 0,39**
13 WEEKS	Tip	29	18.6 ± 1.91
	Mid	41	15.6 ± 0.99
	Base	31	21.5 ± 1.21
	Non-polar	112	21.7 ± 0.63
	Total / Average	**213**	**20,07 ± 0.52**

The effect of different dark incubation periods and explant origins can also be compared in terms of average leaf length during transfer to soil. Accordingly, at 13 weeks of dark incubation period, mid explant differs from base and non-polar whereas it is same as tip origined calli in terms of average leaf length. Also, mid explant origined calli resulted in a lower leaf length under 13 weeks of dark incubation period with respect to 6 weeks and 9 weeks (Figure 3.18).

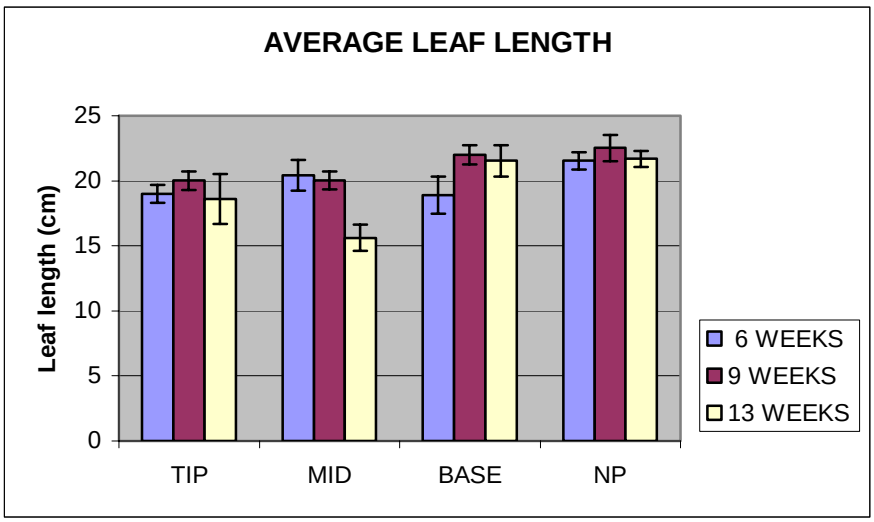


Figure 3.18. Average leaf length data.

In order to monitor the differences associated with dark incubation periods, the data can be pooled and evaluated cumulatively. The comparisons with pooled data revealed that there is no significant difference between different dark incubation periods in terms of average leaf length (Figure 3.19). The average leaf length data during soil transfer shows that at all dark incubation treatments, the explants start to initial embryo germinations nearly during same times and result in similar growth patterns once regenerated.

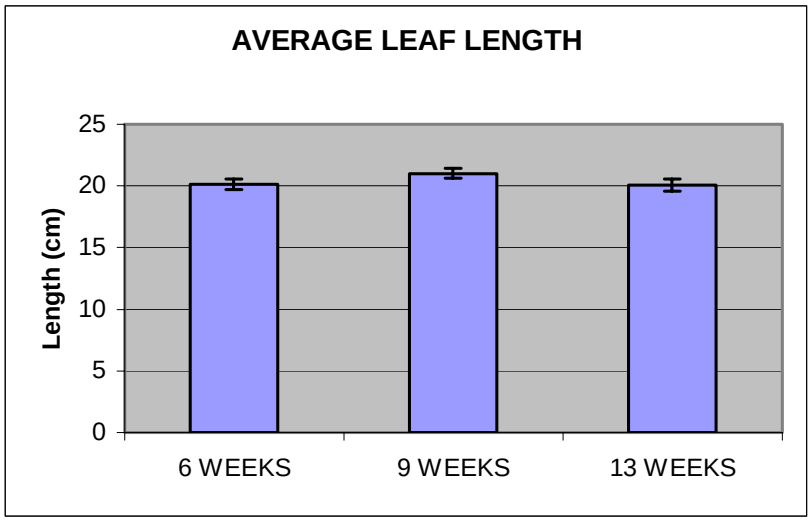


Figure 3.19. Average leaf length with pooled data.

3.1.5. Spike and Seed Characteristics

Possession of high number of seeds with superior quality is one of the most important characteristics of a regenerated plant. The appearance of spike formations in regenerated plants was demonstrated in Figure 3.20.

Figure 3.20. The seed setting of regenerated plants.

In order to monitor these traits, the number of spikes produced by a single plant and number of seeds along with their weights were recorded. The data for total number of spikes and seeds is given in Table 3.6.

Table 3.6. Total number of spikes and seeds.

		Total number of spikes	Total number of seeds
6 WEEKS	Tip (15 plants)	59	309
	Mid (7 plants)	19	178
	Base (6 plants)	23	102
	Non-polar (13 plants)	54	393
	Total	**155**	**982**
9 WEEKS	Tip (1 plant)	2	8
	Mid (7 plants)	15	42
	Base (1 plant)	7	42
	Non-polar (2 plants)	5	32
	Total	**29**	**124**
13 WEEKS	Tip (0 plant)	NA	NA
	Mid (2 plants)	2	4
	Base (2 plant)	2	4
	Non-polar (3 plants)	5	23
	Total	**9**	**32**
OVERALL	**TOTAL**	**193**	**1138**

The spikes were phenotypically normal and they contained seeds. The appearances of spikes and seeds are illustrated in Figure 3.21.

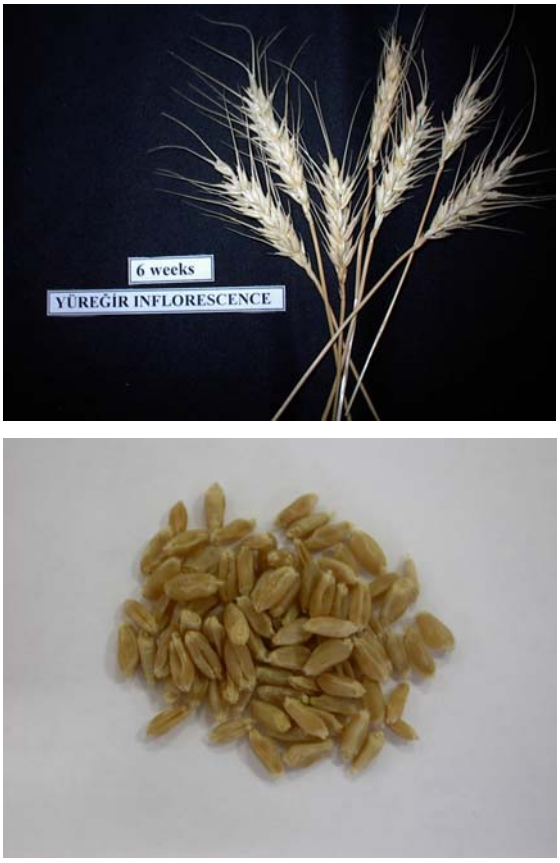


Figure 3.21. Spike and seed of regenerated plants.

The seed settings of explants originated from different regions and incubated at different dark incubation periods can also be demonstrated as the average seed number per spike. The data is shown in Table 3.7.

Table 3.7. Average number of seed per spike.

		Average number of seed per spike
6 WEEKS	Tip	6.07 ± 1.11
	Mid	9.37 ± 1.31
	Base	4.39 ± 0.92
	Non-polar	7.16 ± 0.61
	Average	**6.61 ± 0.53**
9 WEEKS	Tip	4.0 ± 1.0
	Mid	3.0 ± 0.48
	Base	6.0 ± 1.65
	Non-polar	6.4 ± 0.93
	Average	**4.38 ± 0.55**
13 WEEKS	Tip	NA
	Mid	2.0 ± 1.0
	Base	2.0 ± 1.0
	Non-polar	4.6 ± 1.4
	Average	**3.44 ± 0.90**

When average seed numbers were compared, there was no significant difference between different explant regions of 6 weeks dark incubated calli and 13 weeks dark incubated calli. For 9 weeks dark treatment, mid explants were found to have lower number of seeds per spike than base and non-polar origined calli ($p= 0.032$ and $p= 0.003$ respectively in 95 % confidence interval). When the effect of dark treatment was considered, bases, non-polars and tips are statistically same at every dark incubation period; however, mid 6 differs significantly from mid 9 with $p= 0$ and from mid 13 with 0.091. The changes with respect to dark incubation periods

are demonstrated in Figure 3.22. It was seen that average seed number per spike is statistically the same in all of the dark incubation periods with p= 0.079.

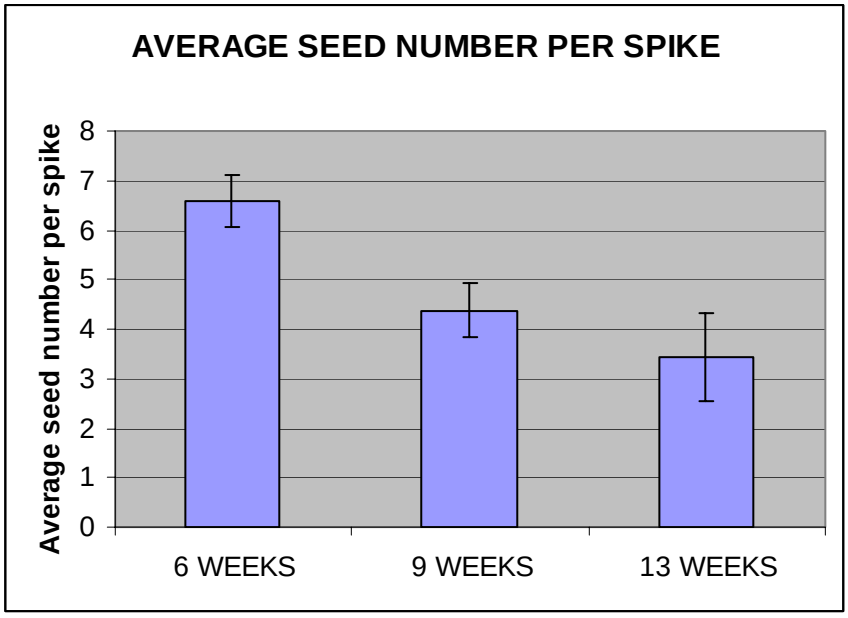


Figure 3.22. Average seed number per spike with pooled data.

Besides the number of seeds present in a spike, their quality is also very important. One of the first parameter associated with seed quality is its weight. Since the number and weight of the seeds are one of the traits that determine the grain yield and usually the ultimate goal in wheat production, the regenerated plants are desired to be as good as counterparts which are not originating from tissue culture.

The average weights of 1000 seeds of regenerated plants change between 26.19, 23.39, and 17.01 at 6 weeks, 9 weeks, and 13 weeks of dark incubation period, respectively (Table 3.8). Yüreğir seeds which are grown in pots under the same conditions with the regenerated plants were used as control. The average weight of

1000 seed of control plants was found as 18.8 (n= 141). In literature, 1000 seed weight of Yüreğir was declared as 45-50 g (Çukurova Institute for Agricultural Research, 1992). Our findings from regenerated plants, in fact, should not be thought as deviating from the literature value which is given as the value obtained in field trials. In greenhouse conditions where the plants are grown in pots, it is expected to obtain grain yield lower than field values.

Table 3.8. Average seed weight

		n	**Average weight of 1000 seeds (g)**
6 WEEKS	Tip	295	24.5 ± 0.5
	Mid	170	28.3 ± 0.6
	Base	57	28.7 ± 1.1
	Non-polar	287	27.3 ± 0.6
	Total	**809**	**26.19 ± 0.38**
9 WEEKS	Tip	8	38.4 ± 0.2
	Mid	44	22.0 ± 1.5
	Base	42	25.1 ± 1.0
	Non-polar	28	18.8 ± 1.3
	Total	**122**	**23.39 ± 0.84**
13 WEEKS	Tip	NA	NA
	Mid	3	15.6 ± 1.8
	Base	NA	NA
	Non-polar	16	17.3 ± 0.9
	Total	**19**	**17.01 ± 0.78**

It is also possible to compare the effect of explant origin and dark incubation period in terms of seed weight as shown in Figure 3.23. Since number of seeds for 13 weeks treatment was very low, only 6 weeks and 9 weeks dark incubation periods were compared in terms of seed weight. When origin of the explant was considered, in 6 weeks dark incubation period, tip 6 differs from mid 6 and base 6 significantly with p= 0 and p= 0.001 respectively while it is same with non-polar 6. Tip 9 is significantly higher than others with p=0. It is also possible to compare same group of explants with respect to their dark incubation periods. Tip 6 and tip 9 are significantly different from each other. Also, mid 6 is significantly different than mid 9 and mid 13 with p=0 and p=0.00, respectively.

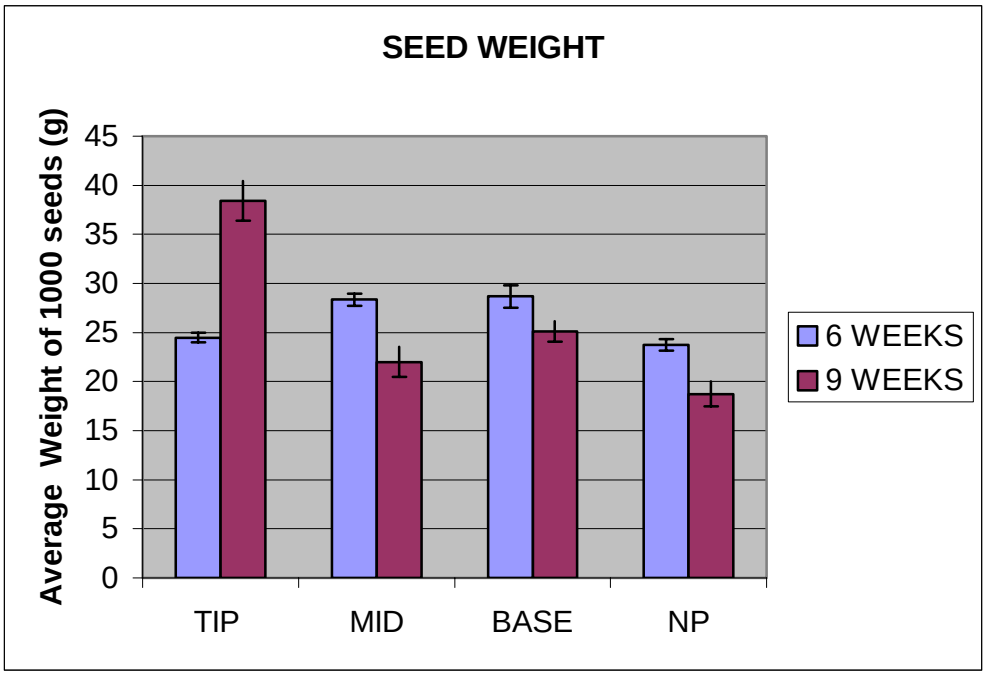


Figure 3.23. Seed weight comparison.

When average seed weights were compared with pooled data, it can be seen that as dark incubation period increases, the seed weight value decreases (p=0, Figure 3.24). Although it might be originated from the insufficient number of seeds especially for 13 weeks of dark incubation (n = 809 for 6 weeks, n= 122 for 9 weeks, and n= 19 for 13 weeks), the trend was found to be significant according to statistical analysis. In this case, it can be concluded that the dark incubation period should not increase 6 weeks in order to obtain high yield and the selection scheme in transformation studies should be constructed accordingly.

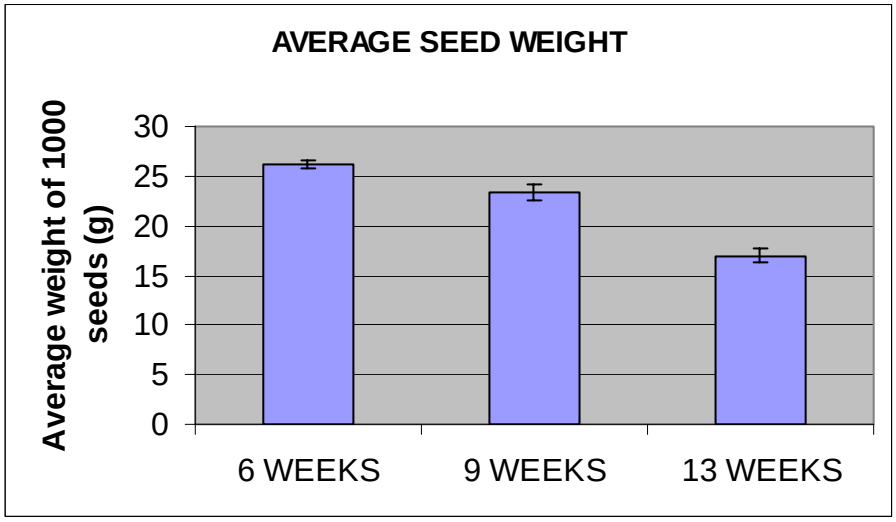


Figure 3.24. Average seed weight by using pooled data.

3.1.6. Correlation between regeneration parameters

The correlations between different regeneration parameters were measured by using Pearson product moment correlation coefficient which is used to measure the degree of linear relationship between two variables. The correlation coefficient

assumes a value between -1 and +1. If one variable tends to increase as the other decreases, the correlation coefficient is negative. Conversely, if the two variables tend to increase together the correlation coefficient is positive.

Correlation analysis can be performed with data for tip, mid and base in order to monitor any correlations associated with different explant origins. The trend in the changes of responses with different dark incubation periods was tried to be correlated. As shown in Table 3.9, 3.10, and 3.11; no significant relationship was detected between any of the parameters. For tip explant, due to the lack of data, correlation coefficients for seed number per spike and seed weight could not be determined. Some of the parameters such as leaf length, seed number per spike, seed weight seem to have a negative correlation with shoot number during soil transfer. Such a behavior might be meaningful due to the lack of resources and competition between tillers; however, since the linear correlation is insignificant, no comments should be added.

Table 3.9. Correlation coefficients of regeneration parameters of tip explants

TIP	**Shoot induction**	**Shoot number (4weeks)**	**Shoot number (soil transfer)**
Seed number per spike	0.931		
p value	0.238		
Shoot number (soil transfer)	0.378	0.014	
p value	0.753	0.991	
Leaf length (soil transfer)	0.464	0.109	0.995
p value	0.693	0.931	0.061

Table 3.10. Correlation coefficients of regeneration parameters of mid explants.

MID	Shoot induction (4weeks)	Shoot number (4weeks)	Shoot number (soil transfer)	Leaf length (soil transfer)	Seed number per spike
Shoot number (4weeks)	0.957				
p value	0.188				
Shoot number (soil transfer)	-0.451	-0.691			
p value	0.702	0.514			
Leaf length (soil transfer)	0.882	0.981	-0.818		
p value	0.312	0.124	0.39		
Seed number per spike	0.227	0.501	-0.972	0.659	
p value	0.854	0.666	0.152	0.542	
Seed weight	0.588	0.798	-0.987	0.899	0.922
p value	0.6	0.412	0.102	0.288	0.254

Table 3.11. Correlation coefficients of regeneration parameters of base explants.

BASE	Shoot induction (4weeks)	Shoot number (4weeks)	Shoot number (soil transfer)	Leaf length (soil transfer)	Seed number per spike
Shoot number (4weeks)	0.64				
p value	0.558				
Shoot number (soil transfer)	-0.182	0.639			
p value	0.883	0.559			
Leaf length (soil transfer)	-0.633	0.19	0.877		
p value	0.564	0.878	0.32		
Seed number per spike	0.76	0.986	0.5	0.021	
p value	0.45	0.108	0.667	0.987	
Seed weight	1	0.626	-0.2	-0.647	0.749
p value	0.011	0.569	0.872	0.552	0.461

Correlation of different parameters of pooled data according to the trend in their changes with respect to different dark incubation periods, namely 6 weeks, 9 weeks, 13 weeks, was demonstrated in Table 3.12.

Table 3.12. Correlation of different parameters with pooled data.

	Shoot induction (4 weeks)	**Shoot induction (8 weeks)**	**Shoot number (4weeks)**	**Shoot number (soil transfer)**	**Leaf length (soil transfer)**	**Seed number per spike**
Shoot induction (8 weeks)	0.999*					
p value	0.034					
Shoot number (4weeks)	1*	1*				
p value	0.019	0.014				
Shoot number (soil transfer)	0.152	0.1	0.122			
p value	0.903	0.936	0.922			
Leaf length (soil transfer)	0.456	0.408	0.429	0.949		
p value	0.699	0.732	0.718	0.204		
Seed number per spike	0.799	0.829	0.817	-0.473	-0.172	
p value	0.411	0.377	0.392	0.686	0.89	
Seed weight	0.981	0.99	0.987	-0.041	0.276	0.899
p value	0.123	0.089	0.104	0.974	0.822	0.288

p values smaller than $\alpha = 0.05$ are highlighted and significant correlation coefficients are indicated with (*).

In the table, the significant correlations were designated with a star. Accordingly, shoot induction values, in terms of shooting frequency, measured at the end of 4 weeks and 8 weeks were found to be significantly correlated positively. As such, the shoot number at the end of 4 weeks was strongly correlated in a positive manner with shooting induction values of 4 weeks and 8 weeks. Other parameters, except seed weight, seem to have a positive correlation; however, they were not significant. Finding insignificant correlations between different parameters do not indicate that there is no correlation at all. Since Pearson product test only measures linear relationships, non-linear relationships between different parameters could not be detected, if there is any. In order to find out these relationships, some other analysis including modeling can be performed, which is beyond the scope of this work.

3.2. *Agrobacterium* Mediated Transformation Studies

3.2.1. Effect of co-cultivation medium on transformation efficiency

The preliminary studies on transformation of wheat inflorescence with *Agrobacterium tumefaciens* AGL I were performed on wheat inflorescence which have been kept at callus induction medium for 1, 17, 18, 29, and 37 days. Following transformation process, calli were co-cultivated with the bacteria on MS_2 medium which contains MS salts and 2 mg/L 2,4-D. At the end of co-cultivation period, histochemical GUS assay was performed and no blue points were detected on explants which were kept on MS_2 for 1, 17, 18, and 37 days. Only a few blue regions which were very faint were detected with 29 days old calli as shown in Figure 3.25.

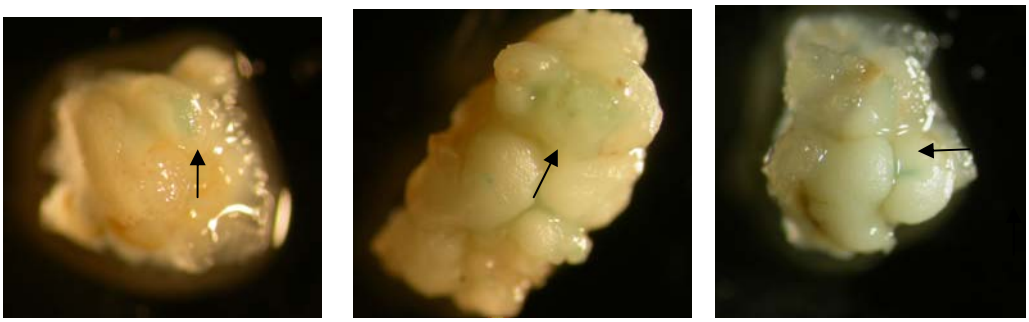


Figure 3.25. Fair blue regions on 29d. old calli co-cultivated on MS_2 medium.

Following these unsuccessful results, some revisions on transformation procedure were considered. Explants, except 29 days old calli, had been rinsed prior to co-cultivation. In subsequent transformation trials, rinsing step was eliminated and calli were just blotted on sterile filter paper and placed on co-cultivation medium. For better expression of the inserted *uid*A gene, after 3 days of co-cultivation, the calli were considered to be further incubated in subsequent transformation experiments.

In order to get an enhanced transformation procedure, an enriched co-cultivation medium was designed. It is known after the report of Tingay and co-workers (1997), who demonstrated successful transformation of barley embryos by a non-supervirulent *Agrobacterium* strain, that inclusion of acetosyringone into co-cultivation medium is very important for the induction of bacterial *vir* genes especially for cereals which are known to be inferior in terms of secreting phenolic compounds. Based on this knowledge, 200 µM acetosyringone was supplemented into co-cultivation medium. Another known phenomenon in transformation of cereals by *Agrobacterium tumefaciens* is the activation of systemic hypersensitivity response and release of hydrogen peroxide which cause necrosis and cell death (Parrott *et al.*, 2002). Ascorbic acid, at 100 mg /L concentration, was added into the medium in order to relieve these symptoms.

First positive signs of transient GUS expression were obtained after utilization of MMD medium in 3 days of co-cultivation period (Figure 3.26). In the figure, the control plants are also shown. They were treated in the same way as the transformed ones except during the bacterial incubation period, they were treated only with MMA medium devoid of *Agrobacterium*. According to these positive signs, it can be concluded that incorporation of phenolic compound acetosyringone and ascorbic acid was essential for successful transformation studies.

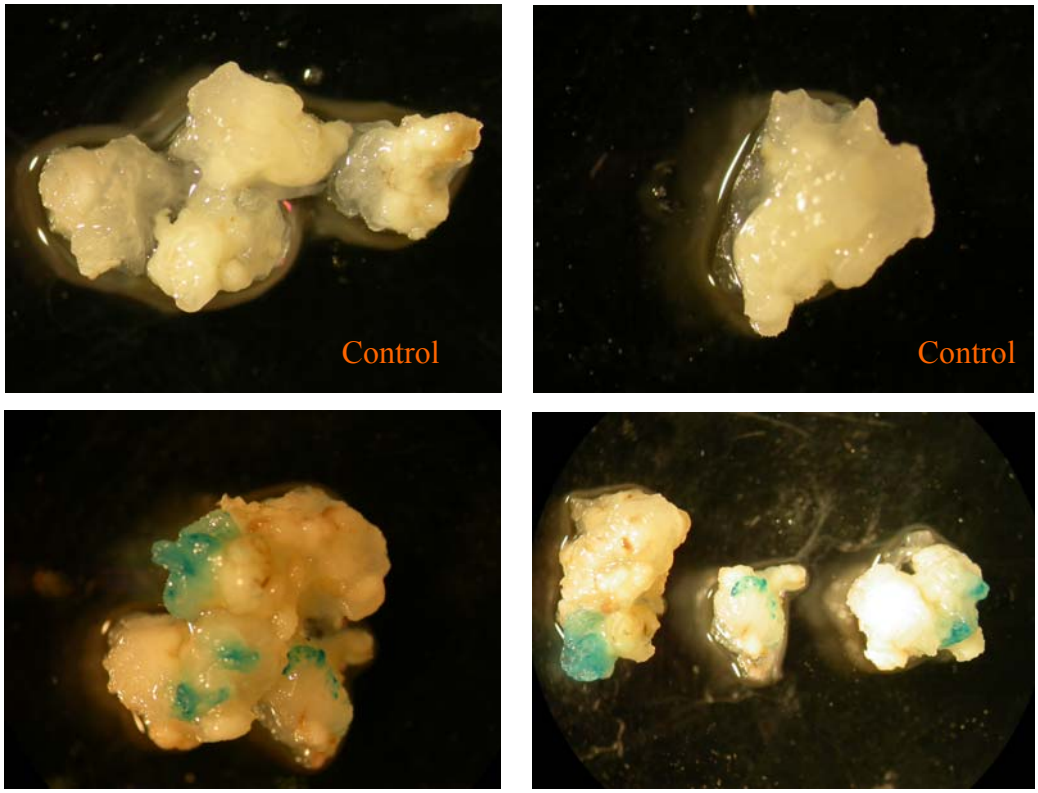


Figure 3.26. Above: Control explants are shown. Below: Transient *uidA* expression after using MMD medium for co-cultivation (29 days old calli)

Besides the positive effects of acetosyringone and ascorbic acid; the reason for not obtaining successful transformants might be the presence of 2,4-D. There might be some negative effects associated with 2,4-D on induction of bacterial *vir* genes or on the activity of enzymes synthesized by these genes. After several transformation experiments, in order to minimize the possible negative effects of 2,4-D, MMD medium was further modified. The 2,4-D concentration on the medium was decreased to 0.5 mg /L (from 1 mg/ L) and pH of the medium was decreased to 5.0 for better action of bacteria. The novel medium was designated as MMM. Promising results were obtained by the utilization of MMM medium (data are given in following sections).

3.2.2. Effect of callus age on transient GUS expression

Agrobacterium-mediated transformation has two biological components; parameters associated with bacterial counterpart and with plant system. Thus, besides optimization the conditions for the bacteria; the physiological condition of the explant has equal importance. The age of the explant is one of the most important determinants which alter the physiological situation of the explant.

Transformation studies were conducted with 1 day old inflorescence and 9, 15, 21, 28, 29, 30, 32, 60, 66 days old calli (Table 3.13). 28-29 days were found to be the period during which plant cells are receptive to *Agrobacterium* infection. Cells can be more prone to transformation not only due to their competence in terms of physiological situation at that developmental stage, but also due to their ability to better cope with the hypersensitivity response when compared to younger explants. The extensive necrosis observed in one day old calli is due to higher surface area to volume ratio of these explants (Figure 3.27). In this manner, using immature inflorescence derived calli rather than freshly isolated immature inflorescence seems to be wiser. In the study of Amoah and co-workers, the optimum age of inflorescence derived calli was found as 21 days (Amoah *et al.*, 2001). The

difference of our findings with theirs is understandable due to genotypic differences resulting from utilization of different cultivars.

Table 3.13. GUS Frequency with respect to callus age.

DAYS IN CALLUS INDUCTION MEDIUM	GUS EXPRESSION FREQUENCY*	GUS EXPRESSION FREQUENCY (only fine blue regions are considered)
1 day	4/32 **	0
9 days	0	0
15 days	0/57	0
21 days	1/40	1/40
28 days	10/60	1/60
28 days	1/35	0
29 days	5/50	3/50
30 days	3/70	0
60 days	0/10	0
66 days	0	0

(*) Frequency is given as number of GUS expressing explants over total number of explants.

(**)MMM medium was used. In all other transformation experiments, MMD medium was utilized.

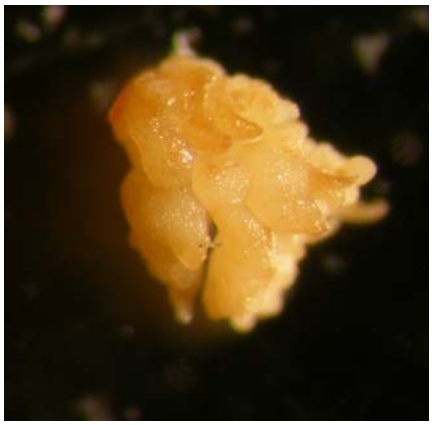


Figure 3.27. Extensive necrosis observed in one day old calli co-cultivated on MMD medium.

3.2.3. Effect of bacterial incubation period

In the utilized transformation procedure, bacteria were centrifuged and re-suspended in MMA medium and incubated for 1 hour at 22°C prior to plant-bacteria encounter. It was thought that the bacterial enzymes which are active during transformation process might not be ready exactly at the end of the 1 h incubation period. Thus, 1.5 h and 2 h incubation durations were also tested. For other species, this period may not be important because during co-cultivation, active bacteria will have opportunity to transfer its DNA. However, since *Agrobacterium*-associated hypersensitivity response is a very big problem in cereals, during the point at which explant is met with bacterial cells, the bacteria should be in its most active and ready state.

Transformation experiments were conducted with 29 days old calli and as the co-cultivation medium, MMM was utilized. At the end of histochemical GUS assay, very distinct blue regions were obtained (Figure 3.28). Transformation frequencies at different bacterial incubation periods are given in Table 3.14.

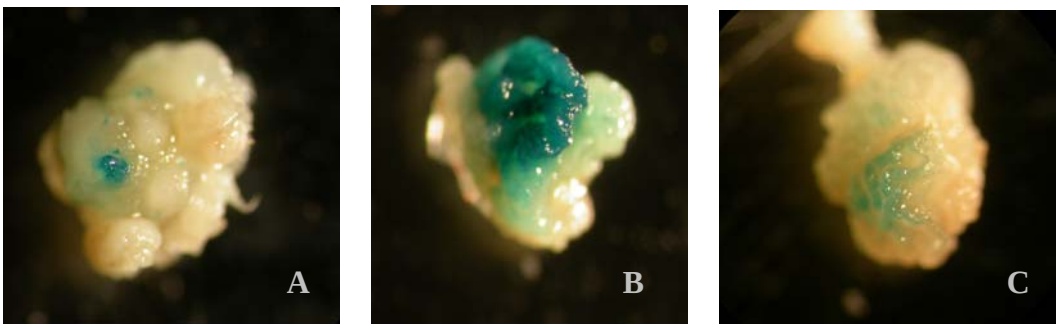


Figure 3.28. GUS expression in 1 h, 1.5 h, and 2 h bacterial induction periods, respectively in A,B,C.

Table 3.14. Effect of bacterial incubation duration

BACTERIAL INCUBATION DURATION	GUS EXPRESSION FREQUENCY	GUS EXPRESSION FREQUENCY (only fine blue regions are considered)
1 h	21/69	7/69
1.5 h	12/90	7/90
2 h	1/56	1/56

As shown in Figure 3.29, 1 h of bacterial incubation duration seems to be most efficient in terms of transient GUS expression. 1 h of bacterial incubation duration results in higher GUS expression frequency than 1.5 h and 2 h, by p= 0.008 and p= 0 respectively. When frequency of GUS expression based on only the distinct blue regions was considered, the mean frequencies were found to be 0.1014, 0.0778, 0.0179 respectively for 1 h, 1.5 h and 2 hours of bacterial incubation period. In this case, frequencies do not differ significantly.

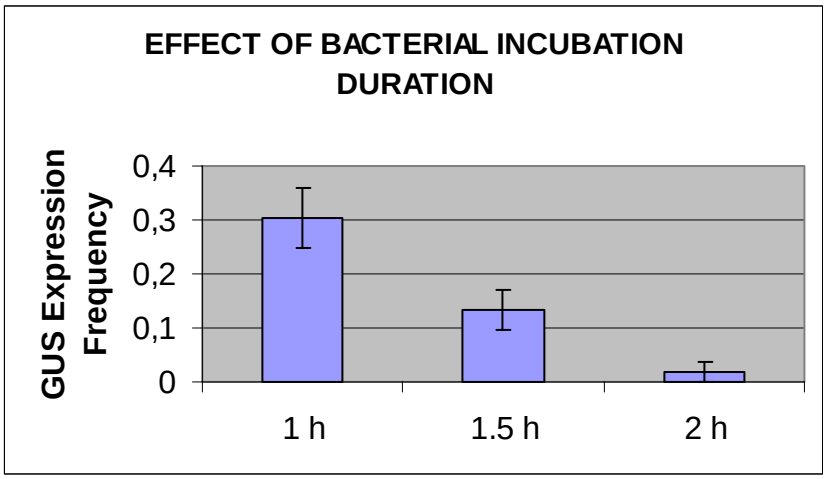


Figure 3.29. Effect of bacterial incubation duration on GUS expression.

3.2.4. Effect of preconditioning

In order to increase transformation efficiency, immature inflorescence derived callus was preconditioned for 2 days prior to transformation process. Preconditioning was performed on MS medium which is supplemented with 100 mg/L ascorbic acid and 1 mg /L 2,4-D. The objective of preconditioning was to prepare the explants for transformation by familiarizing them with ascorbic acid which is also the component of co-cultivation medium.

In contrast to our expectations, GUS expression frequency was dramatically decreased upon preconditioning (Figure 3.30, data are shown on Table 3.15). Incorporation of ascorbic acid might alter the receptivity of explants. Also lowered concentration of 2,4-D which hampers the physiological state of the explant against *Agrobacterium* transformation might be a possible reason for decreased GUS expression frequency.

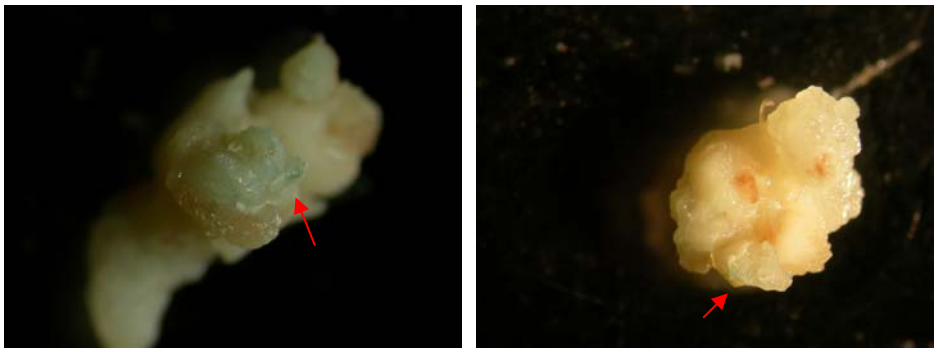


Figure 3.30. Faint blue regions obtained from preconditioned explants.

Table 3.15. Effect of preconditioning

TRIALS*	GUS EXPRESSION FREQUENCY	GUS EXPRESSION FREQUENCY (only fine blue regions are considered)
Set 1	1/73	0/73
Set 2	2/169	0/169

***** Bacterial induction period was 1 h, the calli were co-cultivated on MMM medium.

3.2.5. Effect of vacuum infiltration

All of the data presented until now had been coupled with 600 mmHg vacuum infiltration. In order to determine if this application enhances transformation frequency or it has adverse effects on transformation, transformation experiments without any vacuum infiltration treatment and under 200 mmHg vacuum were performed. The obtained data were shown in Table 3.16.

Table 3.16. GUS expression data for no vacuum infiltration and 200 mmHg vacuum infiltration

VACUUM INFILTRATION	GUS EXPRESSION FREQUENCY	GUS EXPRESSION FREQUENCY (only fine blue regions are considered)
0 mmHg, set 1	16/75	6/75
0 mmHg, set 2	12/96	7/96
200 mmHg, set 1	15/86	1/86
200 mmHg, set 2	83/118	22/118

The GUS expression frequencies of no vacuum infiltration treatment and 200 mmHg vacuum infiltration were compared with that of 600 mmHg vacuum infiltration for which GUS expression frequency was 21/69, and 7 out of 21 were well-defined distinct blue regions. According to this comparison, application of vacuum infiltration at 600mmHg was found to increase transient *uidA* expression from 16 % to 30 % significantly with a p value of 0.014. On the other hand application of a milder vacuum infiltration at 200 mmHg, resulted in even higher GUS expression frequency with a value of 48 % (p=0, Figure 3.31). The reason for obtaining higher GUS expression frequency at -200 mmHg pressure than -600 mmHg pressure may be due to the damage on the plant cells associated with vacuum infiltration process.

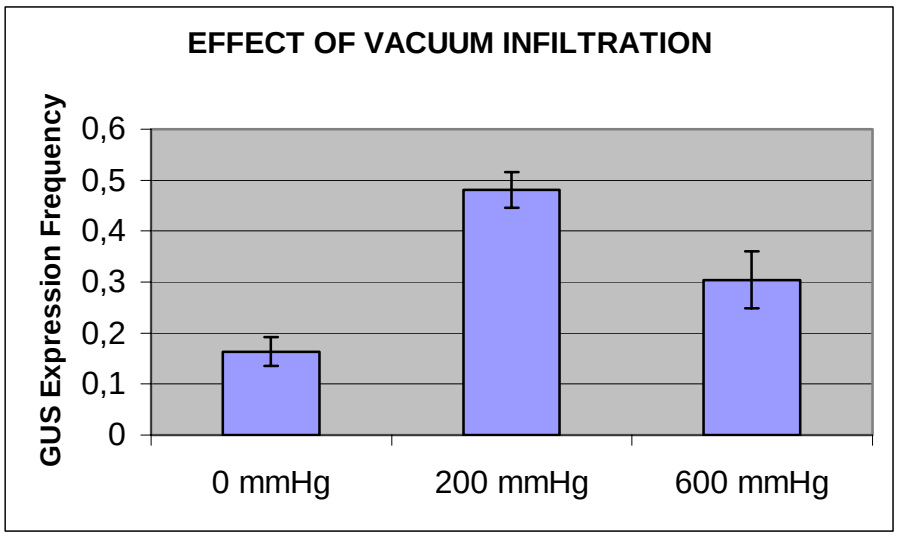


Figure 3.31. Effect of no vacuum infiltration on GUS expression.

When comparisons were performed with the frequency depending only on fine blue regions, the frequency for 0 mmHg was 0.0760; for 200 mmHg frequency was 0.1127 while the frequency for 600 mmHg vacuum infiltration was found to be 0.1014. However, there was no significant difference between them (p= 0.485).

Vacuum infiltration is an effective protocol which alters GUS expression. For example, for lentil, application of vacuum infiltration of *A. tumefaciens* cells at 200 mmHg for 20 minutes was demonstrated to yield the highest transient GUS expression in lentil (Mahmoudian *et al.*, 2002). Obtaining higher GUS expression frequencies with application of vacuum infiltration is also consistent with the findings of Amoah and co-workers (2001), who have reported that vacuum infiltration at 750 mmHg increases levels of GUS expression in immature inflorescence derived calli.

For no vacuum infiltration treatment, although frequency was low, the blue color intensity of the GUS expressing explants was satisfactory as shown below (Figure

3.32). The appearance of 200 mmHg vacuum infiltrated explants was shown in Figure 3.33. The expression of the inserted gene seems very promising.

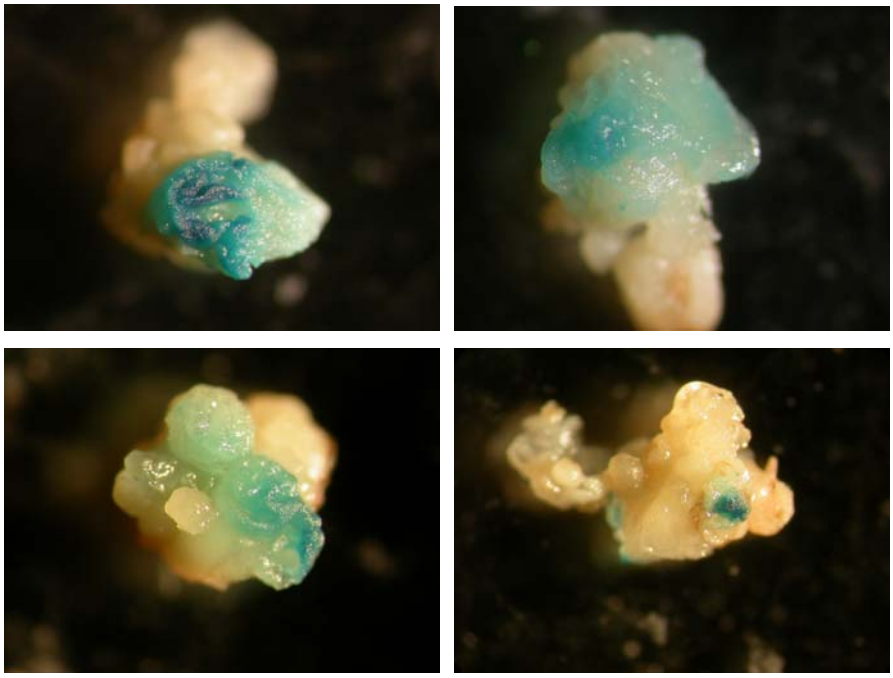


Figure 3.32. Transient GUS expression on explants which are not vacuum infiltrated.

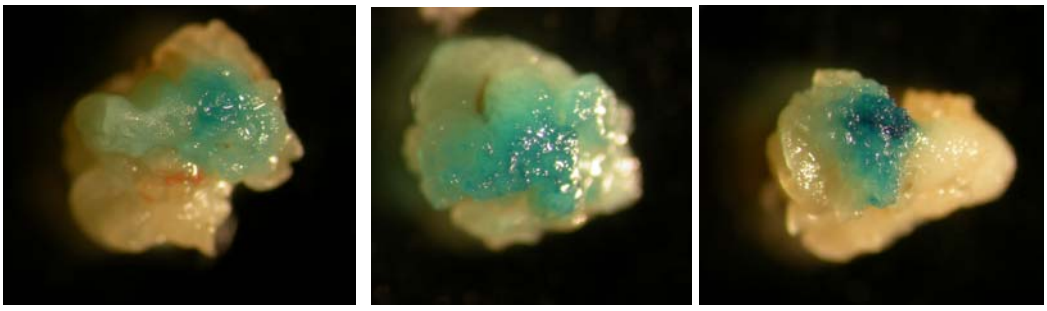


Figure 3.33. Transient GUS expression upon 200 mmHg vacuum infiltration.

Although very distinct GUS expressions were obtained in most of the trials with MMM medium, hypersensitivity associated necrosis still persisted as a big problem. The appearances of explants suffering these symptoms are shown in Figure 3.34. In order to obtain increased transformation efficiencies, strategies focusing in relieving these symptoms should be employed. Utilization of antinecrotic agents such as ascorbic acid and cysteine along with silver nitrate which can also prevent ethylene associated symptoms can be suggested.

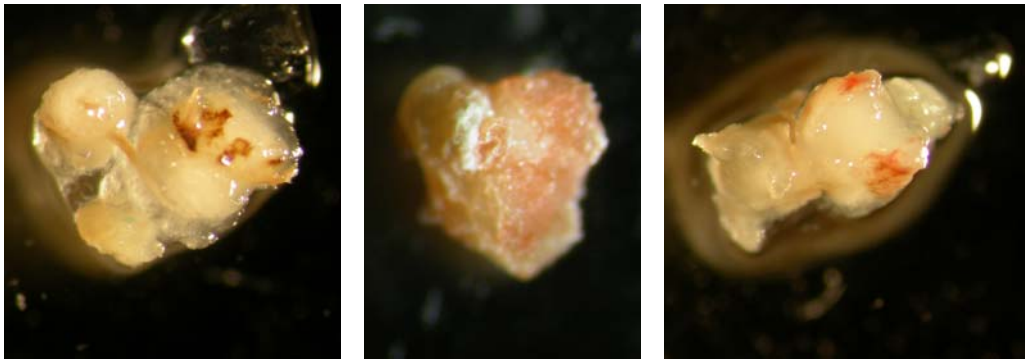


Figure 3.34. Necrosis induced upon transformation procedure.

3.2.6. Determination of selection scheme

Besides having an effective gene delivery system, efficient selection for the transformed cells is extremely important. The plant selection associated with the incorporated gene is *bar* which encodes phosphinothricin acetyl transferase (PAT) and confers resistance to phosphinothricin (PPT) and glufosinate ammonium. Thus, PPT was used as the plant selection agent. In order to determine the optimum concentration of PPT for selecting just the transformed ones but not non-transformants, the growth of wheat calli on 0, 3, 6, 10 mg/L PPT was monitored.

35 days old non-transformed calli, which corresponds approximately to the end of co-cultivation duration, were transferred to PPT containing MS_2 medium and their weight was recorded weekly. The percent changes in callus weight upon different concentrations of PPT applications were demonstrated in Figure 3.35. The appearance of calli was shown in Figure 3.36.

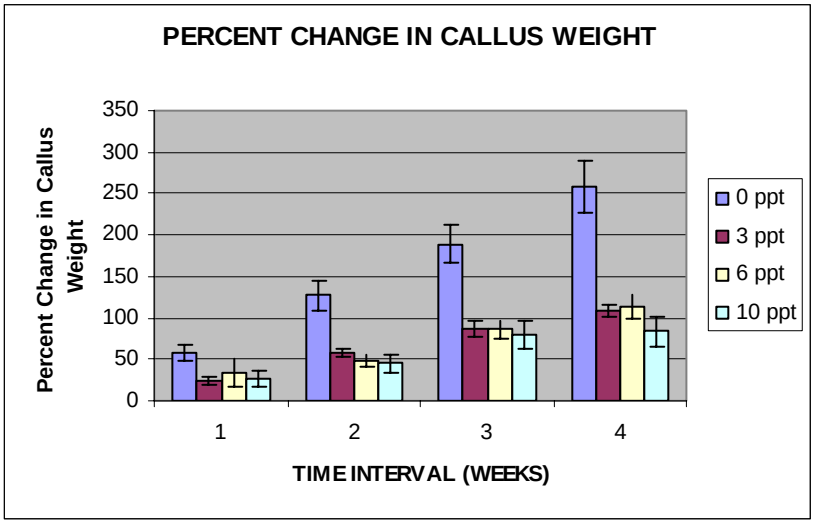


Figure 3.35. Percent changes in callus weight upon PPT application (n=12).

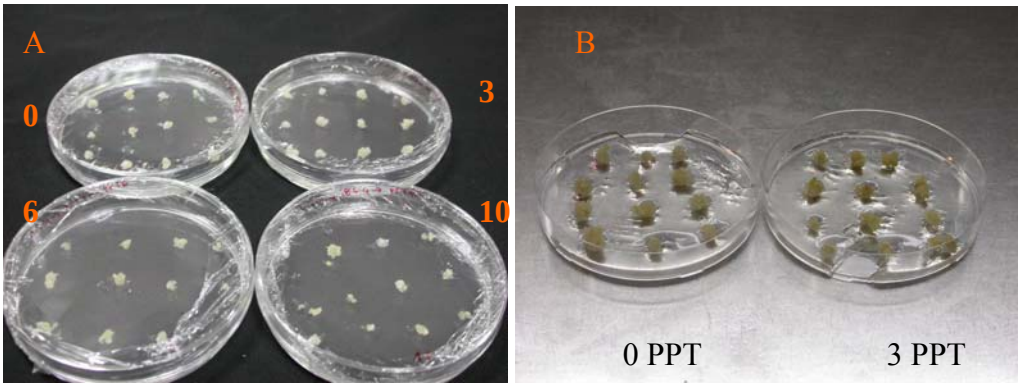


Figure 3.36. A. The appearance of calli at the beginning of PPT experiment. B. Control and 3 PPT application at the end of 5 weeks.

From the graph, it can be seen that even with 3 mg/L PPT application callus growth was retarded with respect to control group. At the end of 4 weeks, the cease in growth was more apparent with 10 mg/ L PPT application. According to these results, when constructing selection scheme for Yüreğir callus, it seems wise to start with 3 mg/L PPT and increase the selection pressure up to 10 mg/L by time.

Transformed calli from one of the sets of 29 days old calli co-cultivated on MMM medium was put onto 3 mg/L PPT, 100 mg/L Cefotaxim containing medium. Cefotaxim was used to eliminate the growth of *Agrobacterium* in the medium. The studies for obtaining stable transformants are still on progress.

Although stable transformants have not obtained yet, current results indicate that even up to 70 % transient gene expression frequency was observed by the optimized protocol (1 h bacterial incubation duration, 200 mmHg vacuum infiltration, MMM co-cultivation medium). According to the literature, by using *Agrobacterium* mediated transformation, transient GUS expression frequency was found between 35 % and 65 % in immature embryo derived calli (Khanna and Daggard, 2003), 52.4 % transient GUS activity was reported by Guo and co-workers (1998) with suspension culture derived calli, and a peak value of 76% GUS expression frequency was obtained with 21 day old calli of immature inflorescence (Amoah *et al.*, 2001). It can be said that by using the Turkish wheat cultivar Yüreğir 89, satisfactory GUS expression frequencies comparable to the literature values were obtained.

CHAPTER IV

CONCLUSION

In this study, the regeneration parameters of immature inflorescence based cultures of Turkish bread wheat cultivar Yüreğir were optimized. The embryogenic capacity and regeneration potential of different regions of inflorescence tissue and the effect of dark incubation period on regeneration potential were documented.

- Every region of immature inflorescence, namely tip, mid, base, had 100 % callus formation frequency and no difference between different regions of the immature inflorescence in terms of callus induction was observed.

- There were no significant changes between tip, mid, and base regions in terms of shoot induction. It can also be concluded that any region of the immature inflorescence can be safely utilized in subsequent transformation studies.

- Prolonged dark incubation period decreased regeneration potential. The regeneration potential of 13 weeks and 16 weeks dark incubated explants were found to be significantly lower than that of 6 weeks and 9 weeks. Six weeks and 9 weeks of dark incubation period seems to be optimum in long term transformation studies.

- It is evident that longer regeneration time enhances the shooting response. The regeneration potential of Yüreğir inflorescence derived calli was

determined as 72.0 %, 64.1%, 26.1 % and 24.3 % for 6, 9, 13, 16 weeks of dark incubation periods, respectively.

- The regeneration of plants from immature inflorescences within 180-260 days is possible. The spikes were phenotypically normal and they produced seeds.

- The shoot number at the end of 4 weeks was found linearly correlated in a positive manner with shoot induction values. No linear correlation was detected between other regeneration parameters.

Along with these regeneration parameters, due to the less time consuming nature of inflorescences during isolation, reduced growth space and time requirements; Yüreğir immature inflorescence is suggested as a good candidate in transformation studies for improvement of Turkish wheat varieties.

Besides regeneration parameters, *Agrobacterium* mediated transformation protocol for Yüreğir immature inflorescence tissue was also optimized by monitoring transient expression of *uid*A gene.

- It can be concluded that incorporation of phenolic compound acetosyringone and ascorbic acid in the co-cultivation medium was essential for successful transformation studies. Better results were obtained with reduced pH (pH=5.0).

- The age of the explant has a paramount importance in transformation success. 3-4 weeks old callus was found to be more receptive to *Agrobacterium* mediated transformation. Using immature inflorescence derived calli rather than freshly isolated immature inflorescence seems to be wiser.

- Vacuum infiltration positively affected the transient expression of *uidA* gene and 200 mmHg vacuum application was found to yield better results.

- When constructing selection scheme for Yüreğir calli, it is suggested to start with 3 mg/L PPT and increase the selection pressure up to 10 mg/L by time.

- *Agrobacterium* associated cell necrosis is still a problem in transformation procedure. In order to obtain increased transformation efficiencies, strategies focusing in relieving these symptoms should be employed.

As the first application of *Agrobacterium* mediated transformation methodology to a Turkish wheat cultivar, in long term, the findings of this study is aimed to be used for enlightening our understanding of *Agrobacterium* mediated transformation in cereals and for obtaining fertile transgenic wheat plants possessing agronomically important genes.

REFERENCES

Ahloowalia B. S. 1982 “Plant regeneration from callus culture in wheat”. Crop Science., 22: 405-410.

Altpeter F., Diaz I., McAuslane H., Gaddour K., Carbonero P., Vasil I.K. 1999 “Increased insect resistance in transgenic wheat stably expressing trypsin inhibitor CMe”. Mol. Breed., 5: 53-63.

Altpeter F., Vasil V., Srivastava V., Vasil I.K. 1996 “Integration and expression of the high-molecular-weight glutenin subunit 1Ax1 gene into wheat”. Nature Biotechnology, 14: 1155-1159.

Amoah B.K., Wu H., Sparks C., Jones H.D. 2001 “Factors influencing *Agrobacterium*-mediated transient expression of *uid*A in wheat inflorescence tissue”. Journal of Experimental Botany, 52: 1135-1142.

Arzani A., Mirodjagh S.S. 1999 “Response of durum wheat cultivars to immature embryo culture, callus induction and *in vitro* salt stress”. Plant Cell, Tissue and Organ Culture, 58:67-72.

Bai D., Knott D.R. 1993 “The effects of level of 2,4-D and time in culture on regeneration rate and chromosome numbers of regenerants from calli of the hybrid *Triticum aestivum* cv. Chinese Spring *ph1b* x *Thinopyrum ponticum* (2n=10x=70)”. Genome, 36:166-172.

Barcelo P., Hagel C., Becker D., Martin A., Lorz H. 1994 “Transgenic Cereal (Tritordeum) Plants Obtained at High-Efficiency by Microprojectile Bombardment of Inflorescence Tissue”. Plant Journal., 5 (4): 583-592.

Barro F., Martin A., Lazzeri P.A., Barcelo P. 1999 “Medium optimization for efficient somatic embryogenesis and plant regeneration from immature inflorescences and immature scutella of elite cultivars of wheat, barley and tritordeum”. Euphytica, 108: 161-167.

Barro F., Rooke L., Bekes F., Gras P., Tatham A.S., Fido R., Lazzeri P.A., Shewry P.R., Barcelo P. 1997 “Transformation of wheat with high molecular weight subunit genes results in improved functional properties”. Nature Biotechnology, 15: 1295-1299.

Benkirane H., Sabounji K., Chlyah A., Chlyah H. 2000 “Somatic embryogenesis and plant regeneration from fragments of immature inflorescences and coleoptiles of durum wheat” Plant Cell Tissue and Organ Cult., 61(2): 107-113.

Bieri S., Potrykus I., Futterer J. 2000 “Expression of active barley seed ribosome-inactivating protein in transgenic wheat”. Theor. Appl.Genet., 100: 755-763.

Blechl A.E., Anderson O.D. 1996 “Expression of a novel high molecular weight glutenin subunit gene in transgenic wheat”. Nat. Biotech., 14: 875-879.

Blechl A.E., Le H.Q., Anderson O.D. 1998 “Engineering changes in wheat flour by genetic transformation”. J. Plant Physiol., 152 : 703-707.

Bliffeld M., Mundy J., Potrykus I., Futterer J. 1999 “Genetic engineering of what for increased resistance to powdery mildew disease”. Theor. Appl. Genet., 98: 1079-1086.

Bommineni V.R., Jauhar P.P. 1996 “Regeneration of plantlets through isolated scutellum culture of durum wheat”. Plant Science, 116: 197-203.

Borelli G.M., Lupotto E., Locatelli F., Wittmer G. 1991 “Long-term optimized embryogenic cultures in durum wheat (*Triticum durum* Desf.)”. Plant Cell Reports., 10: 296-299.

Botti C., Vasil I. K. 1984 “The ontogeny of somatic embryos of *Pennisetum americanum* (L.) in immature inflorescences”. Canad. J. Bot., 62: 1629-1635.

Brinch-Pedersen H., Oleson A., Rasmussen S.K., Holm P.B. 2000 “Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an Aspergillus phytase”. Mol. Breed. 6: 195-206.

Carman J.G. 1995 “Somatic embryogenesis and synthetic seed II” In “Biotechnology in Agriculture and Forestry vol.31”, edited by Y.P.S. Bajaj, Springer, Verlag, Berlin, Heidelberg.

Caswell K. L., Leung N. L., Chibbar R. N. 2000 “An efficient method for in vitro regeneration from immature inflorescence explants of Canadian wheat cultivars”. Plant Cell Tissue and Organ Cult., 60: 69–73.

Chan M., Cheng H., Ho S., Tong W., Xu S. 1993 “*Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α -amylase promoter/β -glucuronidase gene”. Plant Molecular Biology, 22: 491-506.

Chen D.F., Dale P.J. 1992 “A comparison of methods for delivering DNA to wheat: the application of wheat dwarf virus DNA to seeds with exposed apical meristems”. Transgenic Research, 1: 93-100.

Chen W.P., Chen P.D., Liu D.J., Kynast R., Friebe B., Velazhahan R., Muthukrishnan S., Gill B.S. 1999 "Development of wheat scab symptoms is delayed in transgenic wheat plants that constitutively express a rice thaumatin-like protein gene". Theor. Appl. Genet., 99: 755-760.

Chen W.P., Gu X., Liang G.H., Muthukrishnan S., Chen P.D., Liu D.J., Gill B.S. 1998 "Introduction and constitutive expression of a rice chitinase gene in bread wheat using biolistic bombardment and the *bar* gene as a selectable marker". Theor. Appl. Genet., 97: 1296-1306.

Cheng M., Fry J.E., Pang S., Zhou H., Hironaka C.M., Duncan D.R., Conner T.W., Wan Y. 1997 "Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*". Plant Physiology, 115: 971-980.

Chowdhury M.K.U., Vasil V., Vasil I. K. 1994 "Molecular analysis of plants regenerated from embryogenic cultures of wheat (*Triticum aestivum* L.)". Theoretical and Applied Genetics., 87: 821-828.

Clausen M., Krauter R., Schachermayr G., Potrykus I., Sautter C. 2000 "Antifungal activity of a virally encoded gene in transgenic wheat". Nat. Biotech., 18: 446-449.

Cook R.J., Johnson V.A., Allan, R.E. 1993 In "Traditional Crop Breeding: Practices: An historical review to serve as a baseline for assessing the role of modern biotechnology". Organisation for Economic Co-operation and Development, OECD, Paris, p.27-36.

Cornell J. H., Hoveling A.W. 1998 "Wheat: Chemistry and Utilization". Technomic Publishing Company, Lancaster, p.2-4.

Curtis B.C. 2002 “Wheat in the World”. In “Bread Wheat: Improvement and Production”, edited by B.C. Curtis, S. Rajaram, H. Gómez MacPherson, FAO Plant Production and Protection Series No. 30, Food And Agriculture Organization Of The United Nations, Rome.

Çakmak I, Kalaycı M, Ekiz H, Braun H-J, Kılınç Y, and Yilmaz A. 1999. “Zinc-deficiency as a practical problem in plant and human nutrition in Turkey: A NATO-Science for Stability project”. Field Crops Res, 60:175-188.

Dale P.J., Marks M.S., Brown M.M., Woolston C.J., Gunn H., Boulton M.I., Mullineaux P.M., Lewis D.M., Kemp J.M., Chen D.F., Gilmour D.M., Flavell R.B. 1989 “Agroinfection of wheat: Inoculation of *in vitro* grown seedlings and embryos”. Plant Science, 63: 237-245.

De Block M., Debrouwer T., Moens T. 1997 “The development of a nuclear male sterility system in wheat: Expression of the barnase gene under the control of tapetum specific promoters”. Theor.Appl. Genet., 95: 125-131.

Delporte F., Mostade O., Jacquemin J.M. 2001 “Plant regeneration through callus initiation from thin mature embryo fragments of wheat”. Plant Cell, Tissue and Organ Culture, 67: 73-80.

Dodds J.H., Roberts W.L. 1985 “Experiments in Plant Tissue Culture”, 2nd Edition, Cambridge University Press, pp 54-55.

Dölekoğlu T. 2003 “Türkiye’nin Tarım Ürünleri Dış Ticareti”, Tarımsal Ekonomi Araştırma Enstitüsü, TEAE Bakış, sayı 2, nüsha 2.

Durusu İ. Z. 2001 “Optimization of *in vitro* regeneration of Turkish wheat cultivars from immature embryo and inflorescence explants”. Master Thesis, Middle East Technical University, Ankara.

Ertuğrul F. 2002 “Studies on the development of osmotic stress resistant wheat by introducing aldose reductase gene through particle bombardment.” Ph.D. Thesis, Middle East Technical University, Ankara.

Feldman M. 1976 “Wheats”. In Evolution of Crop Plants edited by N.W. Simmonds, Longman, London and New York, pp. 120-128.

Felfoldi K., Purnhauser L. 1992 “Induction of regenerating callus cultures from immature embryos of 44 wheat and 3 triticale cultivars” Cereal Res. Commun., 20: 273–277.

Fennell S., Bohorova N., Ginkel M., Crossa J., Hoisington D. 1996 “Plant regeneration from immature embryos of 48 elite CIMMYT bread wheats”. Theor. Appl. Genet., 92: 163–169.

Fernandez S., Michaux-Ferriere N., Coumans M. 1999 “The embryogenic response of immature embryo cultures of durum wheat (*Triticum durum* Desf.): histology and improvement by $AgNO_3$”. Plant Growth Regulation., 28: 147-155.

Fettig S., Hess D. 1999 “Expression of a chimeric stilbene synthase gene in transgenic wheat lines”. Transgenic Res. 8: 179_/189.

Finer J.J, Finer K.R., Ponappa T. 1999 “Particle bombardment mediated transformation”. Plant Biotechnology, 240: 59-80.

Food and Agricultural Organization of United Nations (FAO) 2002 Reports. http://www.fao.org (Last access date: 28.09.2004).

Gibson L., Benson G. 2002 “Origin, History, and Uses of Oat (*Avena sativa*) and Wheat (*Triticum aestivum*)”. Iowa State University, Department of

Agronomy.http://www.agron.iastate.edu/courses/agron212/Readings/Oat_wheat_history.htm (Last access date: 28.09.2004).

Gill B.S., Friebe B. 2002 "Cytogenetics, Phylogeny and evolution of cultivated wheats". In "Bread Wheat: Improvement and Production", edited by B.C. Curtis, S. Rajaram, H. Gómez MacPherson, FAO Plant Production and Protection Series No. 30, Food And Agriculture Organization Of The United Nations, Rome.

Guo G.Q., Maiwald F., Lorenzen P., Steinbiss H.H. 1998 "Factors influencing T-DNA transfer into wheat and barley cells by *Agrobacterium tumefaciens*". Cereal Res. Commun., 26:15-22.

He D.G., Mouradev A., Yang Y.M., Mouradeva E., Scott K.J. 1994 "Transformation of wheat (*Triticum aestivum* L.) through electroporation of protoplasts". Plant Cell Reports, 14:192-196.

He G.Y., Lazzeri P.A. 1998 "Analysis and optimization of DNA delivery into wheat scutellum and tritordeum inflorescence explants by tissue elctroporation". Plant Cell Reports, 18: 64-70.

He G.Y., Lazzeri P.A. 2001 "Improvement of somatic embryogenesis and plant regeneration from durum wheat (*Triticum turgidum* var. *durum* Desf.) scutellum and inflorescence cultures". Euphytica, 119: 369-376.

He G.Y., Rooke L., Steele S., Bekes F., Gras P., Tatham A.S., Fido R., Barcelo P., Shewry P.R., Lazzeri P.A. 1999 "Transformation of pasta wheat (*Triticum turgidum* L. var durum) with high molecular- weight glutenin subunit genes and modification of dough functionality". Mol. Breed., 5: 377-386.

Hess D., Dressler K., Nimmricher R. 1990 “Transformation experiments by pipetting *Agrobacterium* into the spikelets of wheat (*Triticum aestivum* L.)”. Plant Science, 72: 233-244.

Heun M., Schafer-Pregl R., Klawan D., Castagna R., Accerbi M., Borghi B. and Salamini F. 1997 “Site of Einkorn wheat domestication identified by DNA fingerprinting”. Science, 278: 1312-1314.

Heyser J.W., Nabors M. W., MacKinnon C., Dykes T. A., Demott K. J., Kautzman D. C., Mujeeb-Kazi A. 1985 “Long-term, high frequency plant regeneration and the induction of somatic embryogenesis in callus cultures of wheat (*Triticum aestivum* L.)”. Z. Pflanzenzüchtg., 94: 218-233.

Hiei Y., Ohta S., Komari T., Kumashiro T. 1994 “Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA”. Plant Journal, 6:271-282.

Hoisington. D., Bohorova N., Fennell S., Khairallah M., Pellegrineschi A., Ribaut J.M. 2002 “The application of biotechnology to wheat improvement”. In “Bread Wheat: Improvement and Production”, edited by B.C. Curtis, S. Rajaram, H. Gómez MacPherson, FAO Plant Production and Protection Series No. 30, Food And Agriculture Organization Of The United Nations, Rome.

http://www.thebreadery.com/nutrition_info/nutrition_info.htm (Last access date: 28.09.2004).

Inglett G.E. 1974 “Breeding Genetics”. In “Wheat: Production and Utilization”, edited by George E.Inglett, Avi Publishing Company, Connecticut, p.108-115.

International Grains Council 2003 Grain Market Report. Adopted from Montana Wheat and Barley Committee Web Site http://wbc.agr.state.mt.us/prodfacts/wf/wptwp.html (Last access date: 28.09.2004).

Ishida Y., Saito H., Ohta S., Hiei Y., Komari T., Kumashiro T. 1996 "High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*". Nat. Biotech. 14: 745-750.

Janakiraman V., Steinau M., McCoy S.B., Trick H.N. 2002 "Recent advances in wheat transformation". In Vitro Cell. Dev. Biol.-Plant, 38: 404-414.

Jefferson R.A. 1987 "Assaying chimeric genes in plants: The GUS gene fusion system". Plant Mol. Biol. Rep., 5: 387-405.

Jenes B., Moore H., Cao J., Zhang W., Wu R. 1993 "Techniques for gene transfer". In. Transgenic plants, vol. 1, Engineering and utilization, edited by S. Kung, R. Wu, Academic Press, New York, NY, USA, pp.125-126.

Kapila J., De Rycke R., Montagu M.V., Angenon G. 1997 "An *Agrobacterium*-mediated transient gene expression system for intact leaves". Plant Science, 122: 101-108.

Karunarante S., Sohn A., Mouradov A., Scott J., Steinbiss H.H., Scott K.J. 1996 "Transformation of wheat with the gene encoding the coat protein of barley yellow mosaic virus". Aust. J. Plant Physiol., 23: 429-435.

Khanna H.K., Daggard G.E. 2001 "Enhanced shoot regeneration in nine Australian wheat cultivars by spermidine and water stress treatments". Australian Journal of Plant Physiology, 28: 1243-1247.

Khanna H.K., Daggard G.E. 2003 "*Agrobacterium tumefaciens* mediated transformation of wheat using a superbinary vector and a polyamine-supplemented regeneration medium". Plant Cell Rep. 21: 429-436.

Kikkert J.R. 1993 "The Biolistic PDS-1000/ He device". Plant Cell Tissue and Organ Culture, 33: 221-226.

Kirby E.J.M. 2002 "Botany of the Wheat Plant". In "Bread Wheat: Improvement and Production", edited by B.C. Curtis, S. Rajaram, H. Gómez MacPherson, FAO Plant Production and Protection Series No. 30, Food And Agriculture Organization Of The United Nations, Rome.

Kloti A., Iglesias V.A., Wijnn J., Burkhardt P.K., Datta S.K., Potrykus I. 1993 "Gene transfer by electroporation into intact scutellum cells of wheat embryos". Plant Cell Reports, 12: 671-675.

Leckband G., Lörz H. 1998 "Transformation and expression of a stilbene synthase gene of *Vitis vinifera* L. in barley and wheat for increased fungal resistance". Theor. Appl. Genet., 97: 1004-1012.

Londsdale D.M., Önde S., Cuming A. 1990 "Transient expression of exogenous DNA in intact viable wheat embryos following particle bombardment". Journal of Experimental Botany, 41: 1161-1165.

Lörz H., Baker B., Schell J. 1985 "Gene transfer to cereal cells mediated by protoplast transformation". Molecular and General Genetics, 199: 178-192.

Lu C., Vasil I. K., Ozias-Akins P. 1982 “Somatic embryogenesis in *Zea mays* L.”. Theor. Appl. Genet. 62: 109-112.

Machii H., Mizuno H., Hirabayashi T., Li H., Hagio T. 1998 “Screening wheat genotypes for high callus induction and regeneration capability from anther and immature embryo cultures”. Plant Cell Tissue and Organ Culture, 53: 67-74.

Maddock S.E., Lancaster V.A., Risiott R., Franklin J. 1983 “Plant regeneration from cultured immature embryos and inflorescences of 25 cultivars of wheat (*Triticum aestivum*)”. Journal of Experimental Botany., 34(144): 915-926.

Mahmoudian M., Yücel M., Öktem H.A. 2002 “Transformation of lentil (L*ens culinaris* M.) cotyledonary nodes via vacuum infiltration of *Agrobacterium tumefaciens*”. Plant Mol. Biol. Rep., 20: 251-257.

Mathias R. J., Simpson E. S. 1986 “The interaction of genotype and culture medium on the tissue culture responses of wheat *(Triticum aestivum* L. em. thell) callus”. Plant Cell Tissue Organ Cult. 7:31–37.

McCormac A.C., Wu H., Bao M., Wang Y., Xu R., Elliot M.C., Chen. D.F. 1998 “The use of visual marker genes as cell-specific reporters of *Agrobacterium*-mediated T-DNA delivery to wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.)”. Euphytica, 99: 17-25.

Miller T. 1987 “Systematics and evolution”. In “Wheat breeding: Its scientific basis” edited by F.G.H. Lupton. Chapman and Hall, New york, pp. 15-20.

Mooney P.A., Goodwin P.B., Dennis E.S., Lewellys D.J. 1991 “*Agrobacterium-tumefaciens* gene transfer into wheat tissues”. Plant Tissue and Organ Culture, 25: 209-218.

Murashige T., Skoog F. 1962 “A revised medium for rapid growth and bioassays with tobacco tissue cultures”. Physiol. Plant., 15: 473-479.

Oleson B. T. 1994 In “Wheat production, properties and quality” Chapter 1, edited by Bushuk W. and Rasper V. Blackie Academic and Professional Publ., Londan, England, pp. 1-12.

Ou-Lee T.M., Turgeon R., Wu R. 1986 “Expression of a foreign gene linked to either a plant virus or a Drosophila promoter, after electroporation of protoplasts of rice, wheat and sorghum”. Proceedings of the National Academy of Science of the United States of America, 83: 6815-6819

Özgen M., Türet M., Altınok S., Sancak C. 1998 “Efficient callus induction and plant regeneration from mature embryo culture of winter wheat (*Triticum aestivum* L.) genotypes”. Plant Cell Reports., 18: 331-335.

Özgen M., Türet M., Avcı M. 2001 “Cytoplasmic effects on the tissue culture response of callus from winter wheat mature embryos”. Plant Cell, Tissue and Organ Culture, 64: 81-84.

Özgen M., Türet M., Özcan S., Sancak C. 1996 “Callus induction and plant regeneration from immature and mature embryos of winter durum wheat genotypes”. Plant Breed., 115: 455–458.

Ozias-Akins P., Vasil I. 1982 “Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* L. (wheat): Evidence for somatic embryogenesis”. Protoplasma., 110: 95-105.

Ozias-Akins P., Vasil I. K. 1983 “Improved efficiency and normalization of somatic embryogenesis in *Triticum aestivum* (wheat)”. Protoplasma., 117, 40-44.

Parrott D.L., Anderson A.J., Carman J.G. 2002 "*Agrobacterium* induces plant cell death in wheat (*Triticum aestivum* L.)", Physiol. Mol. Plant Pathol. 60: 59-69.

Patnaik D., Khurana P. 2001 "Wheat biotechnology: A minireview". Electronic Journal of Biotechnology [online]. Vol.4 No. 2, Issue of August 15, 2001. Available from: http://www.ejbiotechnology.info/content/vol4/issue2/full/4/. ISSN: 0717-3458. (Last access date: 28.09.2004).

Pellegrineschi A., Brito R.M., McLean S., Hoisington D. 2004 "Effect of 2,4-dichlorophenoxyacetic acid and NaCl on the establishment of callus and plant regeneration in durum and bread wheat". Plant Cell, Tissue and Organ Culture, 77: 245-250.

Pellegrineschi A., McLean S., Salgado M., Velazquez L., Hernandez R., Brito R.M., Noguera M., Medhurst A., Hoisington D. 2001 "Transgenic wheat plants: a powerful breeding source". Euphytica, 119: 135-138.

Peña R.J. 2002 "Wheat for bread and other uses". In "Bread Wheat: Improvement and Production", edited by B.C. Curtis, S. Rajaram, H. Gómez MacPherson, FAO Plant Production and Protection Series No. 30, Food And Agriculture Organization Of The United Nations, Rome.

Rakszegi M., Tamas C., Szücs P., Tamas L., Bedö Z. 2001 "Current status of wheat transformation". J.Plant Biotechnology, 3 (2): 67-81.

Ranhotra, G. S. 1994 In "Wheat production, properties and quality" Chapter 2,. edited by Bushuk W. and Rasper V., Blackie Academic and Professional Publ., Londan, England, pp.12-24.

Rasco-Gaunt S., Riley A., Barcelo P., Lazzeri P.A. 1999 “Analysis of particle bombardment parameters to optimise DNA delivery into wheat tissues”. Plant Cell Reports, 19: 118-127.

Rashid H., Yokoi S., Toriyama K., Hinata K. 1996 “Transgenic plant production mediated by *Agrobacterium* in Indica rice”. Plant Cell Rep., 15 : 727-730.

Redway F. A., Vasil V., Lu D., Vasil I. K. 1990 “Identification of callus types for long-term maintenance and regeneration from commercial cultivars of wheat *(Triticum aestivum* L.)”. Theor. Appl. Genet., 79: 609–617.

Ritchie S.W., Hodges T. K. 1993 In “Transgenic Plants Volume 1: Engineering and Utilization”, edited by S. Kung and R. Wu, Academic Press Inc., San Diego, New York, Boston, London, Sydney, Tokyo and Toronto, pp 147-173.

Rooke L., Bakes F., Fido R., Barro F., Gras P., Tatham A.S., Barcelo P., Lazzeri P.A., Shewry P.R. 1999 “Overexpresssion of gluten protein in transgenic wheat results in greatly increased dough strength”. J. Cereal Sci. 30: 115-120.

Sahrawat A.K., Becker D., Lütticke S., Lörz H. 2003 “Genetic improvement of wheat via alien gene transfer, an assestment”. Plant Science, 165: 1147-1168.

Sanford J.C., Klein T.M., Wolf E., Allen N. 1987 “Delivery of substances into cells and tissues using a particle bombardment process”. Particulate Science and Technology, 5:27-37.

Schmidt J.W. 1974 “Breeding Genetics”. In “Wheat: Production and Utilization” edited by George E.Inglett, Avi Publishing Company, Connecticut, p.8.

Sears R.G., Deckard E. L. 1982 “Tissue culture variability in wheat: callus induction and plant regeneration”. Crop Sci. 22: 546–550.

Sharma V.K., Rao A., Varshney A., Kothari S.L. 1995 "Comparison of developmental stages of inflorescence for high frequency plant regeneration in *Triticum aestivum* L. and *Triticum durum* L. Desf.". Plant Cell Rep. 15: 227–231.

Sivamani E., Bahieldin A., Wraith J.M., Al-Niemi T., Dyer W.E., Ho T.H.D., Qu R. 2000 "Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene". Plant Sci.155: 1-9.

Sivamani, Brey C.W., Dyer W.E., Talbert L.E., Qu R. 2000 "Resistance to wheat streak mosaic virus in transgenic wheat expressing the viral replicase (NIb) gene". Mol. Breed. 6:469_/477.

Sorokin A.P., Ke X.Y., Chen D.F., Elliott M.C. 2000 "Production of fertile transgenic wheat plants via tissue electroporation". Plant Science, 156: 227-233.

Srivastava V., Anderson O.D., Ow D.W. 1999 "Single-copy transgenic wheat generated through the resolution of complex integration patterns". Proceedings in National Academy of Science of the United States of America, 96: 11117-11121.

Stöger C., Vaquero, Torres E., Sack M., Nicholson L., Drossard J., Williams S., Keen D., Perrin Y., Christou P., Fischer R. 2000 "Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies". Plant Mol. Biol., 42: 583-590.

Stöger S., Williams S., Christou P., Down R.E., Gatehouse J.A. 1999 "Expression of the insecticidal lectin from snowdrop (*Galanthus nivalis* agglutinin; GNA) in transgenic wheat plants: Effect on predation by the grain aphid *Sitobion avenae*". Mol. Breed. 5: 65-73.

Tan K. 1985 "*Triticum* L." In "Flora of Turkey" edited by P.H. Davis, Edinburgh University Press, vol.9, 245-255.

Takumi S., Murai K., Mori N., Nakamura C. 1999 "Trans-activation of a maize Ds transposable element in transgenic wheat plants expressing the Ac transposase gene". Theor. Appl. Genet. 98: 947-953.

Thomashow M.F., Panagopoulos C., Gordon M.P., Nester E.W. 1980 "Host range of *Agrobacterium tumefaciens* is determined by the Ti plasmid". Nature, 283: 794-796.

Tingay S., McElroy D., Kalla R., Fieg S., Wang M., Thorton S., Brettell R. 1997 "*Agrobacterium tumefaciens*-mediated barley transformation", Plant J., 11: 1369-1376.

Union of Turkish Chambers of Agriculture 2004 Wheat Report, http://www.tzob.org.tr/tzob/tzob_ana_sayfa.htm (Last access date: 28.09.2004).

Vasil I. K. 1987 "Developing cell and tissue culture systems for the improvement of cereal and grass crops". J. Plant. Physiol., 128:193-218.

Vasil V., Brown S.M., Re D., Fromm M.E., Vasil I.K. 1991 "Stably transformed callus lines from microprojectile bombardment of cell suspension cultures of wheat". Biotechnology, 9: 743-747.

Vasil V., Castillo A.M., Fromm M.E., Vasil I.K. 1992 "Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus". Biotechnology, 10: 667-674.

Vasil V., Vasil I. K. 1981 "Somatic embryogenesis in cereals". Bioscience 31 (3): 246-248.

Vasil V., Vasil I.K. 1981 “Somatic embryogenesis and plant regeneration from tissue cultures of pearl millet (*Pennisetum americanum*) and *P.americanum x P.purpureum* hybrid.”. Amer.J.Bot. 68: 864-872.

Vasil V., Vasil I.K. 1982 “Characterization of an embryogenic cell suspension culture derived from inflorescences of *Pennisetum americanum* (pearl millet, Graminae)”. Amer.J.Bot. 69: 1441-1449.

Wang Y.C., Klein T.M., Fromm M., Cao J., Sanford J.C., Wu R. 1988 “Transient expression of foreign genes in rice, wheat and soybean cells following particle bambardment”. Plant Molecular Biology, 11: 433-439.

Weir B., Gu X., Wang M., Upadhyaya N., Elliott A.R., Brettell R.I.S. 2001 “*Agrobacterium tumefaciens* mediated transformation of wheat using suspension cells as a model system and green fluorescent protein as a visual marker”. Aust. J. Plant Physiol., 28: 807-818.

Wheat and Barley Committee, Product Fact, available from http://wbc.agr.state.mt.us/prodfacts/usf/usclass.html#, 2004. (Last access date: 28.09.2004).

Woolston C.J., Barker R., Gunn H., Boulton M.I., Mullineaux P.M. 1988 “Agroinfection and nucleotide sequence of cloned wheat dwarf virus DNA”. Plant Molecular Biology, 11: 35-43.

Xia G.M., Li Z.Y., He C.X., Chen H.M., Brettell R. 1999 “Transgenic plant regeneration from wheat (*Triticum aestivum* L.) mediated by *Agrobacterium tumefaciens*”. Acta Phytophysiol. Sin., 25: 22-28.

Zaghmout O.M.F. 1994 “Transformation of protoplasts and intact cells from slowly growing embryogenic callus of wheat (*Triticum aestivum* L.)”. Theoretical and Applied Genetics, 89:577-582.

Zale J.M., Borchardt-Wier H., Kidwell K.K., Steber C.M. 2004 “Callus induction and plant regeneration from mature embryos of diverse set of wheat genotypes”. Plant Cell, Tissue and Organ Culture, 76: 277-281.

Zhang L., French R., Langenberg W.G., Mitra A. 2001 “Accumulation of barley stripe mosaic virus is significantly reduced in transgenic wheat plants expressing a bacterial ribonuclease”. Transgenic Res., 10: 13-19.

Zhang L., Rybczynski J.J., Langenberg W.G., Mitra A., French R. 2000 “An efficient wheat transformation procedure: transformed calli with long-term morphogenic potential for plant regeneration”. Plant Cell Reports, 19: 241-250.

Zhou H., Stiff C.M., Konzak C.F. 1993 “Stably transformed callus of wheat by electroporation-induced direct gene transfer”. Plant Cell Reports 12: 612-616.

APPENDIX A

INFORMATION ON YÜREĞİR-89

The agronomic information of cultivar Yüreğir-89 is summarized in Farmer's booklet of Çukurova Agricultural Research Institute (1992).

YÜREĞİR - 89

- Ekmeklik, orta erkenci bir çeşittir.
- Çukurova Tarımsal Araştırma Enstitüsü tarafından geliştirilmiştir.
- Kışa ve kurağa orta derecede dayanıklıdır.
- Bitki boyu 90-100 cm olup, yatmaya dayanıklıdır.
- Başakları yarı yatık, seyrek ve beyaz renklidir.
- Kahverengi ve sarı pasa orta derecede dayanıklıdır.
- 1000 dane ağırlığı 45-50 gr. civarındadır.
- Dane beyaz renkli ve ovaldir.
- Ekmeklik kalitesi iyi ve yüksek verimli bir çeşittir.
- Ege ve Akdeniz sahil kuşağına önerilmektedir.

APPENDIX B

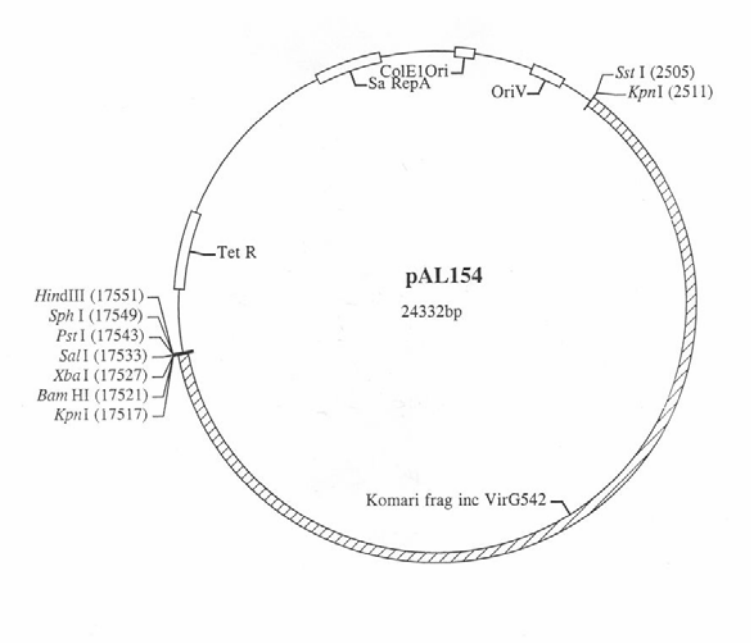
COMPOSITION OF PLANT TISSUE CULTURE MEDIA

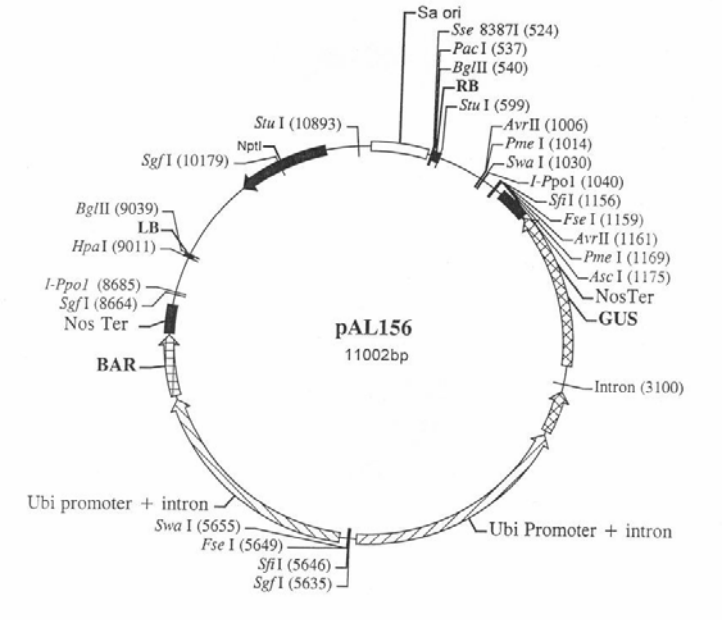
COMPONENT	mg / L
Ammonium nitrate	650.0
Boric acid	6.2
Calciumchloride anhydrous	332.2
Cobalt chloride. $6H_2O$	0.025
Cupric sulfate. 5 H_2O	0.025
Na_2 EDTA	37.26
Ferrous sulfate. 7 H_2O	27.8
Magnesium sulfate	180.7
Manganase sulfate. H_2O	16.9
Molybdic acid (sodium salt). H_2O	0.25
Potassium iodide	0.83
Potassium nitrate	1900.0
Potassiumphosphate monobasic	170.0
Zinc sulfate. 7 H_2O	8.6
Organics	
Glycine (free base)	2.0
Myo-inositol	100.0
Nicotinic acid (free acid)	0.5
Pyridoxine. HCl	0.5
Thiamine. HCl	0.1

4.4 g of powder is utilized to prepare 1 L of medium.

APPENDIX C

PLASMID MAPS





APPENDIX D

TRANSFER AGREEMENT
FOR THE PLASMIDS AND BACTERIA

MATERIAL TRANSFER AGREEMENT
FOR RESEARCH-ONLY PURPOSES

Huseyin Avni Öktem, an employee of Department of Biological Sciences, Middle East Technical University, 06531 Ankara, Turkey, ('the Recipient') wishes to obtain certain tangible materials and/or information from Wendy Harwood & Matthew Perry of the John Innes Centre as described on the reverse side ('the Materials') for the sole purpose of conducting the research specified on the reverse side ('the Research'). The Recipient acknowledges that all rights to the Materials, whether directly or indirectly enclosed therein as well as extracts, replications, summaries, or derivatives thereof, are the sole property of the John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, UK ('JIC') and warrants not to use the Materials for any form of commercial exploitation howsoever.

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3. Not to disclose any information whatsoever with regards to the Material and use thereof, without the prior written approval of JIC.
4. To acknowledge the contribution of [Wendy Harwood & Matthew Perry / JIC / the Biotechnology and Biological Sciences Research Council (BBSRC)] in any publication that may result from use of the Materials.
5. To hold harmless JIC and its governors, officers, employees and agents from any and all liabilities or claims brought by third parties resulting from the transfer to and use of the Materials by the Recipient.
6. This Agreement is personal to the Recipient and not capable of assignment.
7. This Agreement is subject to English Law and exclusive interpretation by the English Courts

Please, have (an) authorized officer(s) of the Middle East Technical University signify the Recipients acceptance of the above by signing and dating two copies of this Agreement and return both copies to Mary Anderson, Contracts Manager, John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, UK. Upon receipt of two completed and executed copies of this Agreement the Materials will be then be sent to the Recipient.

APPENDIX D, CONTINUED

On behalf of and for Middle East Technical University	Huseyin Avni Öktem
Date: March 26, 2003	Date: March 25, 2003
Signature:	
Name (print): AHMET ACAR	HÜSEYIN AVNI ÖKTEM
Title: PROF. DR., VICE-PRESIDENT	PROF. DR

LIST OF THE MATERIALS

Plasmid pAL156, developed in the Crop Genetics department by D. Lonsdale.
pAL156 is an *Agrobacterium tumefaciens* binary vector carrying the BAR selectable marker and the GUS reporter genes.

Plasmid pAL154, developed in the Crop Genetics department by D. Lonsdale.
pAL154 is an *Agrobacterium tumefaciens* binary vector; a derivative of pSoup carrying the Kumari fragment.

DESCRIPTION OF THE RESEARCH

The material will be used in the optimisation of *Agrobacterium*-mediated transformation conditions for local wheat cultivars. Experiments will be conducted on mature & immature embryos, immature inflorescence and callus explants.

APPENDIX E

BACTERIAL CULTURE MEDIA

Yeast Extract Broth Medium (1L)

Nutrient broth	13.5 g
Yeast extract	1 g
Sucrose	5 g
$MgSO_4.7H_2O$ (2 mM)	0.485 g

The pH of the medium was adjusted to 7.2.

YEB + MES Medium (1L)

Nutrient broth	13.0 g
Yeast extract	1 g
Sucrose	5 g
$MgSO_4.7H_2O$	0.493 g
MES (10 mM)	2.132 g

The pH of the medium was adjusted to 5.6. After autoclaving the medium at 121°C for 20 minutes, sterile acetosyringone was added to the medium (final concentration is 20 μM).

APPENDIX F

HISTOCHEMICAL GUS ASSAY SOLUTIONS

GUS Substrate Solution

$NaPO_4$ buffer, pH = 7.0	0.1 M
EDTA, pH = 7.0	10 mM
Potassium ferricyanide, pH = 7.0	0.5 mM
Potassium ferrocyanide, pH = 7.0	0.5 mM
X-Gluc	1.0 mM
Triton X-100	0.1 %

GUS Fixative Solution

Formaldehyde	10 % (v/v)
Ethanol	20 % (v/v)
Acetic acid	5 % (v/v)